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(54) Title: PANCREATIC DUCTAL TROPHIC FACTORS, USES AND COMPOSITION RELATED THERETO

(57) Abstract

The present invention relates to a pancreatic ductal trophic factor (PDTF), or combination of factors, which is expressed from pancreatic embryonic mesenchyme cells, and to cell lines which express these factor(s). Methods of inducing outgrowth of pancreatic duct tissue by contacting the duct tissue with said factor(s), a composition containing the factor(s), or with cells expressing the factor(s) also are disclosed. Cells lines expressing PDTF can be used to screen compounds for activity which affects pancreatic tissue growth. Compositions containing PDTF can be used to treat pancreatic disorders, and to induce outgrowth of pancreatic tissue which then can be activated and/or implanted in a patient.

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Pancreatic Ductal Trophic Factors, Uses and Composition Related Thereto

Background of the Invention

During the early stages of embryogenesis, cells are totipotent and are capable of multidirectional differentiation. As development proceeds, the totipotent cells become determined and committed to differentiate into a given specialized cell type. Final differentiation is associated with the acquisition of specialized cell functions. Thus, the differentiated somatic cells maintain their specialized features throughout the life span of the organism, probably through sustained interactions between the genome and its microenvironment and cell-cell interactions (DiBerardino et al., (1984) Science 224:946-952; Wetts and Fraser, (1988) Science 239:1142-1144; Fisher, (1984) PNAS 81:4414-4418).

Because of the tremendous potential of progenitor cells to differentiate into distinct lineages, there has existed a desire in the art for a continuous source of these isolated pluripotent progenitor cells. For instance, the pluripotent progenitor cells could be extremely useful in the treatment of different disorders that are characterized by insufficient or abnormal functioning of the fully differentiated cells in a given organ, as for example in the human pancreas.

The need for progenitor cells which can form functioning, glucose-responsive pancreatic cells is particularly important. Insulin-dependent diabetes mellitus (IDDM) is a disease characterized by elevated blood glucose and the absence of the hormone insulin. The cause of the raised sugar levels is insufficient secretion of the hormone insulin by the pancreas. In the absence of this hormone, the body's cells are not able to absorb sugar from the blood stream in normal fashion, and the excess sugar accumulates in the blood. Chronically elevated blood glucose damages tissues and organs. IDDM is treated with insulin injections. The size and timing of insulin injections are influenced by measurements of blood sugar.

There are over 400 million diabetics in the world today. For instance, diabetes is one of the most prevalent chronic diseases in the United States, and a leading cause of death. Estimates based on the 1993 National Health Interview Survey (NHIS) indicate that diabetes has been diagnosed in 1% of the U.S. population age <45 years, 6.2% of those age 45-64 years, and 10.4% of those age >65 years. In other terms, in 1995 an estimated 8 million persons in the United States were reported to have this chronic condition. In addition, based on the annual incidence rates for diabetes, it is estimated that about 625,000 new cases of diabetes are diagnosed each year, including 595,000 cases of non-

insulin-dependent diabetes mellitus (NIDDM) and 30,000 cases of insulin-dependent diabetes mellitus (IDDM).

The total cost of diabetes in the United States has been estimated at $92 billion annually, including expenditures on medical products, hospitalization and the value of lost work. Substantial costs to both society and its citizens are incurred not only for direct costs of medical care for diabetes, but also for indirect costs, including lost productivity resulting from diabetes-related morbidity and premature mortality. Persons with diabetes are at risk for major complications, including diabetic ketoacidosis, end-stage renal disease, diabetic retinopathy and amputation. There are also a host of less directly related conditions, such as hypertension, heart disease, peripheral vascular disease and infections, for which persons with diabetes are at substantially increased risk.

While medications such as injectable insulin and oral hypoglycemics allow diabetics to live longer, diabetes remains the third major killer, after heart disease and cancer. Diabetes is also a very disabling disease, because medications do not control blood sugar levels well enough to prevent swinging between high and low blood sugar levels, with resulting damage to the kidneys, eyes, and blood vessels.

Studies have documented that medical costs for persons with diabetes are higher because they visit physician's offices, hospital outpatient departments, and emergency rooms more frequently than their nondiabetic counterparts, and are more likely to be admitted to the hospital. Americans with diabetes have two to five times higher per capita total medical expenditures and per capita out-of-pocket expenses than people without diabetes. These expenses and their associated loss of productivity have impact not only on diabetic patients and their families, but on federal and state governments and society as a whole.

Data from the Diabetes Control and Complications Trial (DCCT) show that intensive control of blood glucose significantly delays complications of diabetes, such as retinopathy, nephropathy, and neuropathy, compared with conventional therapy consisting of one or two insulin injections per day. Intensive therapy in the DCCT included multiple injection of insulin three or more times per day or continuous subcutaneous insulin infusion (CSII) by external pump. Insulin pumps are one of a variety of alternative approaches to subcutaneous multiple daily injections (MDI) for approximating physiological replacement of insulin.

Although it is possible to transplant the human pancreas, the shortage of donors and problems of immune rejection limit this procedure to selected patients. β-cell transplantation has been accomplished successfully in humans, but the large number of β-cells required has been an obstacle.

Summary of the Invention

The present invention relates to a pancreatic ductal trophic factor or combination of factors (hereinafter "PDTF") which are capable of stimulating growth and/or differentiation of pancreatic tissue. As described in further detail below, the subject PDTF can be derived from or expressed by embryonic pancreatic mesenchymal cells. The invention also relates to substantially pure preparations of the subject factor, methods for purifying the subject factor, from mesenchymal cells, and cell lines which produce the factor.

In certain embodiments, the PDTF preparation includes a protein component having an apparent molecular weight in range of 40-50Kd, though this may be a dimeric form of the factor, which protein component is heat stable but proteolysis-sensitive.

In certain embodiments, the PDTF preparation includes one or more TGFβ proteins, e.g., selected from the TGFβ sub-family, the activin sub-family, the decapentaplegic sub-family, the 60A sub-family, gross differentiation factor 1 (GDF-1), GDF-3/VGR-2, dorsalin, nodal, mullerian-inhibiting substance (MIS), and glial-derived neurotrophic growth factor (GDNF). In preferred embodiments, the TGFβ protein(s) is from the TGFβ sub-family, e.g., TGFβ1, TGFβ2, TGFβ3, TGFβ4 or TGFβ5, or a TGFβ encodable by a nucleic acid which hybrdidizes, e.g., under stringent conditions, to a gene encoding a TGFβ of the TGFβ sub-family.

Preferred TGFβ polypeptides correspond to a mature TGFβ protein, or to a biologically active fragment thereof. The TGFβ peptide is preferably a mammalian TGFβ, e.g., encoded by a mammalian TGFβ gene, and even more preferably a human TGFβ protein. In certain embodiments, the TGFβ polypeptide will be a TGFβ5 polypeptide, e.g., preferably at least 50 percent identical with an amino acid sequence of SEQ ID NO: 2, and more preferably at least 70, 80, 85, 90 or 95 percent identical. In certain embodiments, the TGFβ polypeptide can be encoded by a nucleic acid that hybridizes to SEQ ID NO:1, preferably under stringency conditions including a wash step of 5.0 x SSC at 50°C, and even more preferably under stringency conditions including a wash step of 2.0 x SSC at 65°C to 0.2 x SSC at 65°.

In one embodiment, the progenitor cells of the present invention are characterized by an ability for self-regeneration in a culture medium and differentiation to pancreatic lineages. In a preferred embodiment, the progenitor cells are inducible to differentiate into pancreatic islet cells, e.g., β islet cells, α islet cells, δ islet cells, or ϕ islet cells. Such pancreatic progenitor cells may be characterized in certain circumstances by the expression of one or more of: homeodomain type transcription factors such as PDX-1 (STF-1); PAX

gene(s) such as PAX6; PTF-1; hXBP-1; HNF genes(s); villin; tyrosine hydroxylase; insulin; glucagon; and/or neuropeptide Y. The pancreatic progenitor cells of the present invention may also be characterized by binding to lectin(s), and preferably to a plant lectin, and more preferably to peanut agglutinin. In a preferred embodiment, the PDTF composition can induce the expansion/proliferation of PDX-1 expressing cells.

Another aspect of the invention provides a conditioned medium (CM) generated by a culture of fetal pancreatic mesenchymal cells which express one or more PDTFs. The invention also provides a purified or semi-purified preparation of one or more PDTF factors expressed by the fetal cells and purified from the medium. In a preferred embodiment, the PDTFs are substantially pure preparations, purified from e14.5 fetal pancreatic mesenchymal cells.

The pancreatic mesenchymal cells of the present invention which produce PDTFs are mammalian embryonic cells which are characterized by their ability to produce PDTFs. In a certain preferred embodiment, the cells are embryonic 14.5 day (e14.5) murine pancreatic mesenchymal cells. In other embodiments, the pancreatic mesenchymal cells are derived from other mammals, e.g., humans or other primates, of a similar developmental stage, e.g., 9-12 weeks in human fetus.

Another aspect of the invention comprises contacting a PDTF, a composition containing PDTF or a cell which produces PDTF, with pancreatic duct cells under conditions sufficient to induce outgrowth of the pancreatic duct tissue thereby forming pancreatic and/or endocrine tissue.

In yet another embodiment, the invention features pharmaceutical compositions comprising a PDTF or combination of PDTFs, or a cellular component comprising a substantially purified population of embryonic pancreatic mesenchymal cells which produce PDTF and which are capable of proliferation in a culture medium.

In another aspect, the invention features a method for treating a disorder characterized by insufficient insulin activity in a subject, comprising introducing into the subject a pharmaceutical composition comprising PDTF, a preparation of PDTF-producing pancreatic mesenchymal cells, or differentiated cells which have been stimulated using PDTF or PDTF-producing cells, and a pharmaceutically acceptable carrier. In a preferred embodiment the disorder is an insulin dependent diabetes, e.g., type I diabetes.

Another aspect of the invention provides a conditioned media generated by a culture of embryonic pancreatic mesenchyme cells which express a PDTF. The invention also provides a purified or semi-purified preparation of one or more PDTF factors expressed by the embryonic cells and purified from the media. In a currently preferred embodiment, the cells are murine e14.5 pancreatic mesenchymal cells.

Application of the invention can be used to generate new pancreatic tissue for ex vivo implantation, for example from a sample of the patient's own pancreatic tissue. In this method, pancreatic tissue removed from the patient is contacted with PDTF, a composition containing PDTF or with cells which secrete PDTF under conditions sufficient to induce outgrowth of the pancreatic tissue thereby forming new pancreatic tissue. The new tissue then can be transplanted back into the patient. In one embodiment, the pancreatic tissue can be activated prior to implantation, for example by application of PYY peptide or an analog or agonist thereof which induces glucose sensitivity in pancreatic tissue. Methods of ex vivo therapy per se are well-established and do not form a part of this invention.

In a preferred embodiment the subject is a mammal, e.g., a primate, e.g., a human.

Another aspect of the present invention provides a substantially pure population of viable stem cells, which cells have been genetically modified to ectopically express a hematopoietic gene, the expression of which confers a self-renewable phenotype on the stem cell. In certain embodiments, the stem cells, or the progeny thereof, can be formulated into pharmaceutical composition.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are described in the literature. See, for example, Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); DNA Cloning, Volumes I and II (D. N. Glover ed., 1985); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No: 4,683,195; Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al. eds.), Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

Figures 1A-1D are photographs showing the stimulation of outgrowth of PDX-1 expressing cells by a feeder layer of mesenchyme cells in (A) wildtype cells. magnified 4x; (B) wildtype cells magnified 20x; (C) PDX-1/LacZ cells, magnified 4x; and (D) PDX-1/LacZ cells, magnified 20x.

Figures 2A-2D are photographs showing the stimulation of outgrowth of PDX-1 expressing cells from adult pancreatic duct by a feeder layer of e14.5 mesenchyme cells in (A) wildtype ductal explant tissue plus DMEM; (B) PDX-1/LacZ ductal explant plus e14.5 mesenchyme cells, magnified 4x; (C) PDX-1/LacZ ductal explant plus e14.5 mesenchyme cells, magnified 10x; and (D) PDX-1/LacZ ductal explant plus e14.5 mesenchyme cells, magnified 20x.

Figure 3 is a graph illustrating the proliferative response of various ductal strain in the presence of FBS and CM.

Figure 4 is graph illustrating the effect of FBS and CM on proliferative D10 cells;

Figure 5 is a graph illustrating the effect of CM on proliferation of Duct-10 cells;

Figures 6A-6D are photographs showing the effect of conditioned media (A) magnified 4x; (B) magnified (10x); and flow through (C) magnified 4x; and (D) magnified 10x; on the outgrowth of adult pancreatic duct;

Figure 7 is a graph illustrating the effect of pancreatic mesenchyme cells and conditioned media on the outgrowth of pancreatic duct tissue.

Figures 8A-8J are photographs showing the ability of 314.5 feeder layer subclones to stimulate outgrowth from adult pancreatic ductal explants;

Figure 9 is a graph showing the effect of conditioned medium factions on outgrowth of adult rat pancreatic ductal tissue;

Figures 10A-10B are photographs illustrating the effect of (A) DMEM and (B) a fraction E (shown in Figure 9) of the conditioned medium on the outgrowth of adult rat pancreatic duct tissue.

Figure 11 is a graph illustrating the effect of e14.5 conditioned medium fractions on outgrowth of adult mouse explant tissue;

Figure 12 is a graph illustrating the effect of e14.5 PM conditioned medium fractions on outgrowth of PDX-1/LacZ ductal explants;

Figure 13 is a graph showing expression of beta-galactosidase in cell-outgrowths generated from PDX-1/LacZ mice treated with e14.5 fractions of cultured medium;

Figure 14-15D are photographs showing the level of beta-galactosidase expression in PDX-1 explant cells cultured with e14.5 fractionated conditioned medium.

Figure 16 are micrographs demonstrating that boiled conditioned medium retains proliferation activity.

Figures 17 and 18 are graphs illustrating that TGFβ proteins can induce growth of pancreatic progenitor cells.

Figures 19 and 20 are graphs showing the effect of the addition of TGFβ proteins to PDX-1/LacZ ductal explant cultures on LacZ expression.

Figure 21 is a micrograph showing that addition of TGFβ proteins to PDX-1/LacZ ductal explant cultures.

Figures 22 is a micrograph showing that addition of TGFβ proteins can induce growth of pancreatic progenitor cells.

Figures 23 and 24 compare the expression of PDX-1 in pancreatic ductal epithelial of mice injected with the subject conditioned media.

Detailed Description of the Invention

I. Overview

It has been discovered that primary cultures of pancreatic cells unexpectedly can be induced to proliferate and to differentiate, that is, to produce increased numbers of pancreatic cells that form islet-like cell clusters ("ICC") containing a high percentage of cells with increased insulin production, by contacting the primary culture cells with an effective concentration of a pancreatic ductal trophic factor (PDTF) described herein. The pancreatic cells generated by the method of the invention, and the progeny thereof, can be used in a variety of applications. These include but are not limited to transplantation or implantation of the engineered cells *in vivo*; screening cytotoxic compounds, growth /regulatory factors, pharmaceutical compounds, etc., *in vitro*; elucidating the mechanism of certain diseases; studying the mechanism by which drugs and/or growth factors operate; and the production of biologically active products, to name but a few. Moreover, the cells can be used therapeutically for treatment of illnesses requiring transplantation of pancreatic cells, especially insulin producing cells.

For instance, proliferating, differentiated ICCs prepared by the subject method can be used for, e.g., transplantation into diabetic subjects, particularly into Type 1 diabetic (human) patients in whom insulin production is compromised in order to alleviate the symptoms of the disease, as well as into animal models of diabetes for the development of human treatment modalities. Expanding such treated pancreatic cultures increases the

supply of functional β-cell islets for transplantation into diabetic subjects in clinically useful numbers.

The method of the invention can be applied, not only to human fetal pancreatic cells, but also to mature (e.g., adult) human pancreatic cells. Nevertheless, the former are highly preferred because the immature immune system of the fetus reduces the likelihood of fetal islet rejection.

Another aspect of the present invention relates to purified and semi-purified, preparations of PDTF, e.g., for use *ex vivo* or *in vivo*, as well as expression vectors for causing ectopic expressions of PDTF. As described herein, PDTF has an apparent molecular weight in range of 40-50Kd, though this may be a dimeric form of the factor, and has a pancreotrophic activity that is heat stable but proteolysis-sensitive. In a preferred embodiment, the PDTF composition can induce the expansion/proliferation of PDX-1 expressing cells.

In a related manner, another aspect of the present invention makes available a conditioned media, including PDTF, e.g., produced by E14.5 cells.

The PDTF-treated cells of the invention, and the progeny thereof, can be used in a variety of applications. These include but are not limited to transplantation or implantation of the engineered cells *in vivo*; screening cytotoxic compounds, allergens, growth/regulatory factors, pharmaceutical compounds, etc., *in vitro*; elucidating the mechanism of certain diseases; studying the mechanism by which drugs and/or growth factors operate; and the production of biologically active products, to name but a few.

II. Definitions

For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

The term "agonist", as used herein, is meant to refer to an agent that upregulates (e.g., mimics potentiates or enhances) at least one TGFβ bioactivity. A TGFβ agonist can be a wild-type TGFβ protein or derivative thereof having at least one bioactivity of a wild-type TGFβ protein or peptidomimetic of TGFβ which functions as an agonist of (e.g., mimics) TGFβ activity. A TGFβ agonist can also be a compound that upregulates expression of a TGFβ gene or which increases at least one bioactivity of a TGFβ protein. A TGFβ agonist therefore includes those agents that upregulate the production and/or secretion of insulin in response to glucose. An agonist can also be a compound which increases the interaction of a TGFβ polypeptide with another molecule, e.g., a TGF receptor, or which mimics the binding to and distortion of a TGFβ receptor by native TGFβ. Another illustrative agonist is a compound which enhances binding of a

transcription factor to the upstream region of a TGFβ or TGFβ receptor gene thereby enhancing the synthesis of the insulin protein. An agonist can also be a compound that upregulates expression of a TGFβ gene or which increases the amount of TGFβ protein present, e.g., by increasing protein synthesis or decreasing protein turnover. Further, a TGFβ agonist can be a TGFβ antagonist inhibitor.

"α cells" are found in the islets of Langerhans in the pancreas. Alpha cells secrete glucagon, a hormone that has effects opposite to those of insulin (it raises blood glucose levels).

As used herein the term "animal" refers to mammals, preferably mammals such as humans. Likewise, a "patient" or "subject" to be treated by the method of the invention can mean either a human or non-human animal.

The terms "β cell" or "pancreatic β cell" are interchangeable as used herein and refer to cells in the pancreatic islets that are of the lineage of cells that produce insulin in response to glucose. β cells are found in the islets of Langerhans in the pancreas. Beta cells secrete insulin in a regulated fashion in response to blood glucose levels. In Type I or insulin dependent diabetes mellitus (IDDM) beta cells are destroyed through an auto-immune process. Since the body can no longer produce endogenous insulin, injections of exogenous insulin are required to maintain normal blood glucose levels.

The term "blood glucose level" refers to the concentration of glucose in blood. The normal blood glucose level (euglycemia) is approximately 120 mg/dl. This value fluctuates by as much as 30 mg/dl in non-diabetics.

As used herein, the term "cellular composition" refers to a preparation of cells, which preparation may include, in addition to the cells, non-cellular components such as cell culture media, e.g. proteins, amino acids, nucleic acids, nucleotides, co-enzyme, anti-oxidants, metals and the like. Furthermore, the cellular composition can have components which do not affect the growth or viability of the cellular component, but which are used to provide the cells in a particular format, e.g., as polymeric matrix for encapsulation or a pharmaceutical preparation.

The term "conditioned media" refers to the supernatant, e.g., free of the cultured cells/tissue, resulting after a period of time in contact with the cultured cells such that the media contains certain proteins or other factors produced by the cells and secreted into the culture.

The term "culture medium" is recognized in the art, and refers generally to any substance or preparation used for the cultivation of living cells. Accordingly, a "tissue culture" refers to the maintenance or growth of tissue, e.g., explants of organ primordia or of an adult organ in vitro so as to preserve its architecture and function. A "cell culture"

refers to a growth of cells in vitro; although the cells proliferate they do not organize into tissue per se.

"Differentiation" in the present context means increased numbers of islet-like cell clusters containing an increased proportion of β-epithelial cells that produce increased amounts of insulin.

By "enhancing differentiation of a cell" is meant the act of increasing the extent of the acquisition or possession of one or more characteristics or functions which differ from that of the original cell (i.e., cell specialization). This can be detected by screening for a change in the phenotype of the cell (e.g., identifying morphological changes in the cell and/or surface markers on the cell).

By "enhancing survival or maintenance of a cell" encompasses the step of increasing the extent of the possession of one or more characteristics or functions which are the same as that of the original cell (i.e., cell phenotype maintenance).

The term "explant" refers to a portion of an organ taken from the body and grown in an artificial medium.

By "ex vivo" is meant cells that have been taken from a body, temporarily cultured in vitro, and returned to a body.

The term "heat stable" means that the designated protein retains substantial activity when heated, e.g., the protein retains at least 75 percent, more preferably at least 80, 85, 90 or even 95 percent of its activity when heated, e.g. when boiled (100°C), for 1 minute, and more preferably 5, 10, 15 or even 20 minutes.

The condition of "hyperglycemia" (high blood sugar) is a condition in which the blood glucose level is too high. Typically, hyperglycemia occurs when the blood glucose level rises above 180 mg/dl. Symptoms of hyperglycemia include frequent urination, excessive thirst and, over a longer time span, weight loss.

On the other hand, "hypoglycemia" (low blood sugar) is a condition in which the blood glucose level is too low. Typically, hypoglycemia occurs when the blood glucose level falls below 70 mg/dl. Symptoms of hypoglycemia include moodiness, numbness of the extremities (especially in the hands and arms), confusion, shakiness or dizziness. Since this condition arises when there is an excess of insulin over the amount of available glucose it is sometimes referred to as an insulin reaction.

The term "impaired glucose tolerance" is used to describe a person who, when given a glucose tolerance test, has a blood glucose level that falls between normal and hyperglycemic. Such a person is at a higher risk of developing diabetes although they are not considered to have diabetes.

The terms "glucose non-responsive" or "glucose non-responsiveness" as used herein describe both the complete inability of cells, islets or animals to respond to treatment with or administration of glucose, as well as decreased responsiveness to glucose (e.g., by cells that do not produce sufficient levels of insulin in response to glucose or that require significantly higher levels of glucose to respond at normal levels).

The term "lineage committed cell" refers to a progenitor cell that is no longer pluripotent but has been induce to differentiate into a specific cell type, e.g., a pancreatic cell.

The term "organ" refers to two or more adjacent layers of tissue, which layers of tissue maintain some form of cell-cell and/or cell-matrix interaction to form a microarchitecture.

The term "pancreas" is art recognized, and refers generally to a large, elongated, racemose gland situated transversely behind the stomach, between the spleen and duodenum. The pancreatic exocrine function, e.g., external secretion, provides a source of digestive enzymes. Indeed, "pancreatin" refers to a substance from the pancreas containing enzymes, principally amylase, protease, and lipase, which substance is used as a digestive aid. The exocrine portion is composed of several serous cells surrounding a lumen. These cells synthesize and secrete digestive enzymes such as trypsinogen, chymotrypsinogen, carboxypeptidase, ribonuclease, deoxyribonuclease, triacylglycerol lipase, phospholipase A_2, elastase, and amylase.

The endocrine portion of the pancreas is composed of the islets of Langerhans. The islets of Langerhans appear as rounded clusters of cells embedded within the exocrine pancreas. Four different types of cells- α, β, δ, and ϕ-have been identified in the islets. The α cells constitute about 20% of the cells found in pancreatic islets and produce the hormone glucagon. Glucagon acts on several tissues to make energy available in the intervals between feeding. In the liver, glucagon causes breakdown of glycogen and promotes gluconeogenesis from amino acid precursors. The δ cells produce somatostatin which acts in the pancreas to inhibit glucagon release and to decrease pancreatic exocrine secretion. The hormone pancreatic polypeptide is produced in the ϕ cells. This hormone inhibits pancreatic exocrine secretion of bicarbonate and enzymes, causes relaxation of the gallbladder, and decreases bile secretion. The most abundant cell in the islets, constituting 60-80% of the cells, is the β cell, which produces insulin. Insulin is known to cause the storage of excess nutrients arising during and shortly after feeding. The major target organs for insulin are the liver, muscle, and fat-organs specialized for storage of energy.

The term "progenitor cell" refers to an undifferentiated cell which is capable of proliferation and giving rise to more progenitor cells having the ability to generate a large

number of mother cells that can in turn give rise to differentiated, or differentiable daughter cells. As used herein, the term "progenitor cell" is also intended to encompass a cell which is sometimes referred to in the art as a "stem cell". In a preferred embodiment, the term "progenitor cell" refers to a generalized mother cell whose descendants (progeny) specialize, often in different directions, by differentiation, e.g., by acquiring completely individual characters, as occurs in progressive diversification of embryonic cells and tissues. As described below, in a preferred embodiment, the progenitor cells of the present invention are pancreatic progenitor cells.

The term "pancreatic progenitor cell" refers to a cell which can differentiate into a cell of pancreatic lineage, e.g. a cell which can produce a hormone or enzyme normally produced by a pancreatic cell. For instance, a pancreatic progenitor cell may be caused to differentiate, at least partially, into α, β, δ, or ϕ islet cell, or a cell of exocrine fate. The pancreatic progenitor cells of the invention can also be cultured prior to administration to a subject under conditions which promote cell proliferation and differentiation. These conditions include culturing the cells to allow proliferation and confluence *in vitro* at which time the cells can be made to form pseudo islet-like aggregates or clusters and secrete insulin, glucagon, and somatostatin.

The term "percent identical" refers to sequence identity between two amino acid sequences or between two nucleotide sequences. Identity can each be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When an equivalent position in the compared sequences is occupied by the same base or amino acid, then the molecules are identical at that position; when the equivalent site occupied by the same or a similar amino acid residue (e.g., similar in steric and/or electronic nature), then the molecules can be referred to as homologous (similar) at that position. Expression as a percentage of homology/similarity or identity refers to a function of the number of identical or similar amino acids at positions shared by the compared sequences. Various alignment algorithms and/or programs may be used, including FASTA, BLAST or ENTREZ. FASTA and BLAST are available as a part of the GCG sequence analysis package (University of Wisconsin, Madison, Wis.), and can be used with, e.g., default settings. ENTREZ is available through the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md. In one embodiment, the percent identity of two sequences can be determined by the GCG program with a gap weight of 1, e.g., each amino acid gap is weighted as if it were a single amino acid or nucleotide mismatch between the two sequences.

The term "phenotype" refers to a particular biologic or biochemical characteristic of a cell, e.g., the cell expresses, or does not express as the case may be, a particular gene or genes (sometimes referred to herein as "marker").

"Primary culture" denotes a mixed cell population of human pancreatic cells that permits interaction of epithelial and mesenchymal cells within ICC. The word "primary" takes its usual meaning in the art of tissue culture.

"Proliferation" indicates an increase in cell number.

The term "proteolysis-sensitive" means that the designated protein retains substantial activity when mixed with a protease, e.g., typsin, chymotrypsin, papain or the like, e.g., the protein retains at least 75 percent, more preferably at least 80, 85, 90 or even 95 percent of its activity when mixed with a protease for 1 minute, and more preferably when mixed with a protease for 10, 30, 60 or even 120 minutes.

As used herein, the term "specifically hybridizes" refers to the ability of a nucleic acid, e.g., a coding sequence of a PDTF, to hybridize to at least 15 consecutive nucleotides of another nucleic acid sequence.

The term "substantially pure population" refers to an isolated population of cells which are made of predominantly of cells having a defined phenotype, e.g., at least 70 percent of the cells in the culture have a given phenotype, more preferably at least 75, 80, 85, 90 or 95 percent of the cells in the culture have a given phenotype, e.g. are PDX-1 positive.

Thus, a superscripted plus sign (+) after a cell marker name indicates a positive phenotype, e.g., the cells produce a statistically significant level of expression of the gene product. Likewise, a superscripted negative sign (-) after a cell marker name indicates a negative phenotype, e.g., the cells do not express a statistically significant level of the corresponding gene product. Finally, a superscripted "neg/low" means a that a particular cell marker is either not expressed at a statistically significant level or is expressed at a level significantly lower than, e.g., a differentiated pancreatic cell, e.g., at level of only 50 or less, more preferably 10 percent or less, and even more preferably 1 percent less than the reference cell.

As used herein the term "substantially pure", with respect to collections of cells, refers to a population of cells that is at least about 75%, preferably at least about 85%, more preferably at least about 90%, and most preferably at least about 95% pure, with respect to a type of cell making up a total cell population. Recast, the term "substantially pure" refers to a population of cells of the present invention that contain fewer than about 20%, more preferably fewer than about 10%, most preferably fewer than about 5%, of

lineage committed cells in the original unamplified and isolated population prior to subsequent culturing.

The term "tissue" refers to a group or layer of similarly specialized cells which together perform certain special functions.

As used herein, the terms "transforming growth factor-beta" and "TGFβ" denote a family of structurally related paracrine polypeptides found ubiquitously in vertebrates, and prototypic of a large family of metazoan growth, differentiation, and morphogenesis factors (see, for review, Massaque et al. (1990) *Ann Rev Cell Biol* 6:597-641; Massaque et al. (1994) *Trends Cell Biol.* 4:172-178; Kingsley (1994) *Gene Dev.* 8:133-146; and Sporn et al. (1992) *J Cell Biol* 119:1017-1021). As described in Kingsley, *supra*, the TGFβ superfamily has at least 25 members, and can be grouped into distinct sub-families with highly related sequences. The most obvious sub-families include the following: the TGFβ sub-family, which comprises at least five genes that are much more similar to TGFβ-1 than to other members of the TGFβ superfamily; the activin sub-family, comprising homo- or hetero-dimers or two sub-units, inhibinβ-A and inhibinβ-B. The decapentaplegic sub-family, which includes the mammalian factors BMP2 and BMP4, which can induce the formation of ectopic bone and cartilage when implanted under the skin or into muscles. The 60A sub-family, which includes a number of mammalian homologs, with osteoinductive activity, including BMP5-8. Other members of the TGFβ superfamily include the gross differentiation factor 1 (GDF-1), GDF-3/VGR-2, dorsalin, nodal, mullerian-inhibiting substance (MIS), and glial-derived neurotrophic growth factor (GDNF). It is noted that the DPP and 60A sub-families are related more closely to one another than to other members of the TGFβ superfamily, and have often been grouped together as part of a larger collection of molecules called DVR (dpp and vg1 related). Unless evidenced from the context in which it is used, the term TGFβ as used throughout this specification will be understood to generally refer to members of the TGFβ superfamily as appropriate. Reference to members of the TGFβ sub-family will be explicit, or evidenced from the context in which the term TGFβ is used.

As described below, in a preferred embodiment, the mesenchyme cells of the present invention are embryonic pancreatic mesenchymal cells.

III. Illustrative Embodiments

The present invention relates to a factor or factors which stimulate outgrowth of pancreatic duct tissue. Several cell lines, mostly of embryonic mesenchymal origin, can stimulate outgrowth of pancreatic duct tissue. These outgrowths were not simply spreading of existing cells (i.e., characterized by BrdU staining), the new material is

pancreatic/endocrine in nature, i.e., characterized by PDX-1 staining (an early marker of pancreatic development expressed in epithelial cells). This stimulation was shown to be able to occur across a transwell, suggesting a soluble factor and a process that does not require cell-cell contact.

PDTFs of the present invention are produced by mammalian embryonic pancreatic mesenchyme cells. The embryonic mesenchyme cells used in the currently preferred embodiment of the present invention are murine in origin, and were derived from a fetal mouse pancreas at day 14.5 of gestation. However, it is understood that embryonic mesenchymal cells of other mammals also can be used, and that the gestation time may vary depending upon the mammal, that is, the developmental stage which the murine fetal pancreas reaches at 14.5 may occur either later or earlier in the gestation cycle in a mammal other than a mouse. Thus, the embryonic mesenchymal cells which can be used in the invention can be any mammalian embryonic mesenchymal pancreatic cells which are at a developmental stage substantially corresponding to day 14.5 in the mouse.

The fetal mesenchymal cells can be isolated from the fetal pancreas and a culture of the cells can be established by any method suitable for isolating cells and establishing cell lines. For example, e14.5 pancreatic mesenchyme cells used in the preferred embodiment of the invention were obtained by removing the fetal pancreas from a mouse embryo on day 14.5 of gestation, treating the pancrease tissue with collagenase, dispase and hyaluronidase to form a cell suspension, filtering the suspension to separate the mesenchyme cells, then plating the mesenchyme cells on a growth medium. The resulting culture contains mesenchyme cells, and may contain other cells, such as epithelial cells.

In a preferred embodiment of the present invention, the culture of e14.5 mesenchyme cells was grown in a serum-free conditioned medium. In this embodiment, the mesenchyme cells first were grown to sub-confluency using a standard complete medium (e.g., DMEM basal medium with 10%FBS and g-INF at 33°C and at 5%CO_2). The conditioned medium was generated by switching the culture to a serum-free medium (e.g., DMEM basal medium either with or without 0.05% BSA) and incubating at 37°C and 5%CO_2. During culture in the conditioned medium, the mesenchyme cells expressed PDTF into the medium. The medium was removed from the cell culture every 24 hours for 3 to 4 days. The media were pooled, and then concentrated by filtering through an ultrafilter having a molecular weight cutoff (MWCO) sufficient to cause PDTF to be retained and thus concentrated in the retentate portion. It was discovered that a filter having a MWCO of about 10,000 was effective for this purpose.

The concentrated conditioned medium then was contacted with pancreatic duct tissue sections. Surprisingly, after a few days exposure to the conditioned media, the pancreatic duct tissue showed significant outgrowth, that is, formation of new pancreatic

tissue. These results, which are shown in the Figures and discussed in detail in the Exemplification, confirm the presence in the media of PDTFs which originated from the embryonic mesenchymal cells.

PDTF has certain physical characteristics, including molecular weight and stability, that are similar to many growth factors, including IGF, TGF, FGF, EGF, HGF, or VEGF, and particularly to members of the TGFβ superfamily. For example, in one embodiment of the invention, conditioned medium obtained from one or more of the present embryonic mesenchymal pancreatic cell lines was concentrated using a filter having a molecular weight cut-off of about 10,000 daltons. The retentate had a higher concentration of PDTF and the filtrate was devoid of activity. Further, the activity in the conditioned medium could be eliminated by protease treatment, but was resistant to 5 minutes of boiling (a slight stimulation even seemed to occur after the latter treatment).

As described in greater detail below, we have tested the effects of various TGF-β proteins on adult ductal explants, and have demonstrated that TGF-β2, 3 and 5 stimulate proliferation of pancreatic progenitor cells in the explant. To determine if there was an expansion/proliferation of PDX-1 expressing cells, ductal expants including a PDX-1/lacZ reporter gene were cultured with TGF-β2, 3 and 5, and we observed expression of the reporter gene in explants treated with the TGF-β proteins.

Thus, in certain embodiments, the PDTF preparation includes one or more TGFβ proteins, e.g., selected from the TGFβ sub-family, the activin sub-family, the decapentaplegic sub-family, the 60A sub-family, gross differentiation factor 1 (GDF-1), GDF-3/VGR-2, dorsalin, nodal, mullerian-inhibiting substance (MIS), and glial-derived neurotrophic growth factor (GDNF). In preferred embodiments, the TGFβ protein(s) is from the TGFβ sub-family, e.g., TGFβ1, TGFβ2, TGFβ3, TGFβ4 or TGFβ5, or a TGFβ encodable by a nucleic acid which hybrdidizes, e.g., under stringent conditions, to a gene encoding a TGFβ of the TGFβ sub-family. Appropriate stringency conditions which promote DNA hybridization, for example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C, are known to those skilled in the art or can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50°C to a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C, to high stringency conditions at about 65°C.

Yet another aspect of the invention pertains to the identification and ultimate preparation of any factors, particularly extracellular factors which are pancreotrophic, therefore produced by embryonic mesenchymal cells and which serve as inductive

molecules for the proliferation and/or differentiations of pancreatic cells. Autocrine and/or paracrine factor(s) which are produced by such cell cultures can be provided as a conditioned media, or the factors can be purified by standard protein purification procedures known in the art.

As described in the appended examples, the embryonic mesenchymal cells, such as E14.5 cells or the like, secrete one or more factors that are able to induce an insulin-producing pancreatic cell. While not wishing to be bound by any particular theory, it is nevertheless noted that our observations suggest that the factor found in conditioned media is a polypeptide which is either secreted as a soluble protein or which is an extracellular portion of a cell membrane protein that is released by cleavage or the like.

Various methods are employed to isolate and purify the inductive factor(s) from conditioned media. To examine the nature of the soluble factors in the conditioned media, the factors can be isolated by such art techniques as may include differential precipitation, molecular sieve chromatography, ion-exchange chromatography, isoelectric focusing, dialysis, gel electrophoresis and affinity and immunoaffinity chromatography. Fractions enriched in the proliferation activity can be identified using an assay based on, for example, the ability of a particular fraction to cause expansion of a normal stem cell culture. Fractions enriched in such activity can be further purified by standard methods until, for example, a single band resulted upon SDS-PAGE. Micro-sequencing and standard cloning techniques can then be used to identify the gene encoding the inductive factor.

Where intended for administration to an animal or addition to a cell culture as an additive, the are a variety of different formulations of the inductive factor from which to chose. As appropriate, the inductive factor is provided in a pharmaceutically acceptable carrier, e.g., which should be substantially inert so as not to act with the active component. Suitable inert carriers include water, alcohol, polyethylene glycol, mineral oil or petroleum gel, propylene glycol and the like.

To prepare the pharmaceutical compositions of this invention, an effective amount of the factor as the active ingredient is combined in intimate admixture with a pharmaceutically acceptable carrier, which carrier may take a wide variety of forms depending on the form of preparation desired for administration. These pharmaceutical compositions are desirable in unitary dosage form suitable, particularly, for administration orally, rectally, percutaneously, or by parenteral injection. For example, in preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed such as, for example, water, glycols, oils, alcohols and the like in the case of oral liquid preparations such as suspensions, syrups, elixirs and solutions; or solid carriers such as starches, sugars, kaolin, lubricants, binders, disintegrating agents and the like in

the case of powders, pills, capsules, and tablets. Because of their ease in administration, tablets and capsules represents the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. For parenteral compositions, the carrier will usually comprise sterile water, at least in large part, though other ingredients, for example, to aid solubility, may be included. Injectable solutions, for example, may be prepared in which the carrier comprises saline solution, glucose solution or a mixture of saline and glucose solution. Injectable suspensions may also be prepared in which case appropriate liquid carriers, suspending agents and the like may be employed. Also included are solid form preparations which are intended to be converted, shortly before use, to liquid form preparations. In the compositions suitable for percutaneous administration, the carrier optionally comprises a penetration enhancing agent and/or a suitable wetting agent, optionally combined with suitable additives of any nature in minor proportions, which additives do not introduce a significant deleterious effect on the skin.

It is especially advantageous to formulate the subject compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used in the specification and claims herein refers to physically discreate units suitable as unitary dosages, each unit containing a predetermined quantity of active ingredient calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. Examples of such dosage unit forms are tablets (including scored or coated tablets), capsules, pills, powders packets, wafers, injectable solutions or suspensions, teaspoonfuls, tablespoonfuls and the like, and segregated multiples thereof.

The pharmaceutical preparations of the present invention can be used, as stated above, for the many applications which can be considered cosmetic uses. Cosmetic compositions known in the art, preferably hypoallergic and pH controlled are especially preferred, and include toilet waters, packs, lotions, skin milks or milky lotions. The preparations may contain, besides the inductive factor, components usually employed in such preparations. Examples of such components are oils, fats, waxes, surfactants, humectants, thickening agents, antioxidants, viscosity stabilizers, chelating agents, buffers, preservatives, perfumes, dyestuffs, lower alkanols, and the like. If desired, further ingredients may be incorporated in the compositions, e.g. anti-inflammatory agents, antibacterials, antifungals, disinfectants, vitamins, sunscreens, antibiotics, or other anti-acne agents.

Examples of oils comprise fats and oils such as olive oil and hydrogenated oils; waxes such as beeswax and lanolin; hydrocarbons such as liquid paraffin, ceresin, and squalane; fatty acids such as stearic acid and oleic acid; alcohols such as cetyl alcohol, stearyl alcohol, lanolin alcohol, and hexadecanol; and esters such as isopropyl myristate, isopropyl palmitate and butyl stearate. As examples of surfactants there may be cited

anionic surfactants such as sodium stearate, sodium cetylsulfate, polyoxyethylene laurylether phosphate, sodium N-acyl glutamate; cationic surfactants such as stearyldimethylbenzylammonium chloride and stearyltrimethylammonium chloride: ampholytic surfactants such as alkylaminoethylglycine hydrocloride solutions and lecithin; and nonionic surfactants such as glycerin monostearate, sorbitan monostearate, sucrose fatty acid esters, propylene glycol monostearate, polyoxyethylene oleylether, polyethylene glycol monostearate, polyoxyethylene sorbitan monopalmitate, polyoxyethylene coconut fatty acid monoethanolamide, polyoxypropylene glycol (e.g. the materials sold under the trademark "Pluronic"), polyoxyethylene castor oil, and polyoxyethylene lanolin. Examples of humectants include glycerin, 1,3-butylene glycol, and propylene glycol; examples of lower alcohols include ethanol and isopropanol; examples of thickening agents include xanthan gum, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, polyethylene glycol and sodium carboxymethyl cellulose; examples of antioxidants comprise butylated hydroxytoluene, butylated hydroxyanisole, propyl gallate, citric acid and ethoxyquin; examples of chelating agents include disodium edetate and ethanehydroxy diphosphate; examples of buffers comprise citric acid, sodium citrate, boric acid, borax, and disodium hydrogen phosphate; and examples of preservatives are methyl parahydroxybenzoate, ethyl parahydroxybenzoate, dehydroacetic acid, salicylic acid and benzoic acid.

For preparing ointments, creams, toilet waters, skin milks, and the like, typically from 0.01 to 10% in particular from 0.1 to 5% and more in particular from 0.2 to 2.5% of the active ingredient, e.g., of the inductive factor, will be incorporated in the compositions. In ointments or creams, the carrier for example consists of 1 to 20%, in particular 5 to 15% of a humectant, 0.1 to 10% in particular from 0.5 to 5% of a thickener and water; or said carrier may consist of 70 to 99%, in particular 20 to 95% of a surfactant, and 0 to 20%, in particular 2.5 to 15% of a fat; or 80 to 99.9% in particular 90 to 99% of a thickener; or 5 to 15% of a surfactant, 2-15% of a humectant, 0 to 80% of an oil, very small (< 2%) amounts of preservative, coloring agent and/or perfume, and water. In a toilet water, the carrier for example consists of 2 to 10% of a lower alcohol, 0.1 to 10% or in particular 0.5 to 1% of a surfactant, 1 to 20%, in particular 3 to 7% of a humectant, 0 to 5% of a buffer, water and small amounts (< 2%) of preservative, dyestuff and/or perfume. In a skin milk, the carrier typically consists of 10-50% of oil, 1 to 10% of surfactant, 50-80% of water and 0 to 3% of preservative and/or perfume. In the aforementioned preparations, all % symbols refer to weight by weight percentage.

Particular compositions for use in the method of the present invention are those wherein the factor is formulated in liposome-containing compositions. Liposomes are artificial vesicles formed by amphiphatic molecules such as polar lipids, for example, phosphatidyl cholines, ethanolamines and serines, sphingomyelins, cardiolipins, plasmalogens, phosphatidic acids and cerebiosides. Liposomes are formed when suitable

amphiphathic molecules are allowed to swell in water or aqueous solutions to form liquid crystals usually of multilayer structure comprised of many bilayers separated from each other by aqueous material (also referred to as coarse liposomes). Another type of liposome known to be consisting of a single bilayer encapsulating aqueous material is referred to as a unilamellar vesicle. If water-soluble materials are included in the aqueous phase during the swelling of the lipids they become entrapped in the aqueous layer between the lipid bilayers.

Water-soluble active ingredients such as, for example, various salt forms of a inductive factor, are encapsulated in the aqueous spaces between the molecular layers. The encapsulation of these proteins can be achieved by a number of methods. The method most commonly used involves casting a thin film of phospholipid onto the walls of a flask by evaporation from an organic solvent. When this film is dispersed in a suitable aqueous medium, multilamellar liposomes are formed. Upon suitable sonication, the coarse liposomes form smaller similarly closed vesicles.

Water-soluble active ingredients are usually incorporated by dispersing the cast film with an aqueous solution of the compound. The unencapsulated compound is then removed by centrifugation, chromatography, dialysis or other art-known suitable procedures. The lipid-soluble active ingredient is usually incorporated by dissolving it in the organic solvent with the phospholipid prior to casting the film. If the solubility of the material in the lipid phase is not exceeded or the amount present is not in excess of that which can be bound to the lipid, liposomes prepared by the above method usually contain most of the material bound in the lipid bilayers; separation of the liposomes from unencapsulated material is not required.

A particularly convenient method for preparing liposome formulated forms of the factor is the method described in EP-A-253,619, incorporated herein by reference. In this method, single bilayered liposomes containing encapsulated active ingredients are prepared by dissolving the lipid component in an organic medium, injecting the organic solution of the lipid component under pressure into an aqueous component while simultaneously mixing the organic and aqueous components with a high speed homogenizer or mixing means, whereupon the liposomes are formed spontaneously.

The single bilayered liposomes containing the encapsulated factor can be employed directly or they can be employed in a suitable pharmaceutically acceptable carrier for localized administration. The viscosity of the liposomes can be increased by the addition of one or more suitable thickening agents such as, for example xanthan gum, hydroxypropyl cellulose, hydroxypropyl methylcellulose and mixtures thereof. The aqueous component may consist of water alone or it may contain electrolytes, buffered systems and other ingredients, such as, for example, preservatives. Suitable electrolytes

which can be employed include metal salts such as alkali metal and alkaline earth metal salts. The preferred metal salts are calcium chloride, sodium chloride and potassium chloride. The concentration of the electrolyte may vary from zero to 260 mM, preferably from 5 mM to 160 mM. The aqueous component is placed in a suitable vessel which can be adapted to effect homogenization by effecting great turbulence during the injection of the organic component. Homogenization of the two components can be accomplished within the vessel, or, alternatively, the aqueous and organic components may be injected separately into a mixing means which is located outside the vessel. In the latter case, the liposomes are formed in the mixing means and then transferred to another vessel for collection purpose.

The organic component consists of a suitable non-toxic, pharmaceutically acceptable solvent such as, for example ethanol, glycerol, propylene glycol and polyethylene glycol, and a suitable phospholipid which is soluble in the solvent. Suitable phospholipids which can be employed include lecithin, phosphatidylcholine, phosphatydylserine, phosphatidylethanol-amine, phosphatidylinositol, lysophosphatidyl-choline and phospha-tidyl glycerol, for example. Other lipophilic additives may be employed in order to selectively modify the characteristics of the liposomes. Examples of such other additives include stearylamine, phosphatidic acid, tocopherol, cholesterol and lanolin extracts.

In addition, other ingredients which can prevent oxidation of the phospholipids may be added to the organic component. Examples of such other ingredients include tocopherol, butylated hydroxyanisole, butylated hydroxytoluene, ascorbyl palmitate and ascorbyl oleate. Preservatives such a benzoic acid, methyl paraben and propyl paraben may also be added.

In another aspect, the present invention facilitates the cloning of genes involved in the inductions of an insulin-producing phenotype by PDTF. By "cloning" it is meant the isolation of a nucleic acid sequence encoding a protein of interest, e.g., from RNA or DNA sources. For instance, the subject cells can be used for the identification, isolation, and study of genes and gene products that are up- or down-regulated by an inductive signal resulting from contact with PDTF. To illustrate, if new transcription is required for the production of one or more induced proteins, a subtractive cDNA library prepared after hybridizing mRNA from control target cells (not contacted with PDTF) to mRNA from experimental target cells (contacted with PDTF) can be used to isolate genes that are turned on or turned off by the presence of PDTF. This type of subtractive approach has been used successfully to isolate a variety of new genes (see, for example, Wang et al. (1991) *PNAS. USA* 88:11505-11509).

A similar approach involves the use of "differential display". See, for example, Liang et al. (1992) *Cancer Res* 52:6966-6998; Liang et al. (1992) *Science* 257:967-971; and Liang et al. (1993) *Nuc Acid Res* 21:3269-3275. The strategy of the method is to use PCR to amplify partial cDNAs that have been made by reverse transcription of RNAs isolated from the two cells of interest, in the case a pancreatic stem cell which is contacted with PDTF and one which is not. The PCR products are then separated in adjacent lanes on a denaturing polyacrylamide gel, and products that are made in one tissue but not in the other are cut directly from the gel, subcloned and sequenced.

Since it is only possible to get good resolution of ~100 PCR products on a single gel, the PCR primers are selected so that only subsets of the RNAs are represented in each PCR reaction. This is accomplished by carrying out the reverse transcription using an oligo-dT primer that consists of 12 T's and two additional 3' bases that confer specificity. This oligo-dT primer is also used in the PCR reactions along with a second primer of arbitrary sequence, which is expected to anneal to different cDNAs at different distances away from the polyA$^+$ tail. By varying the sequence of this second primer and the sequence of the last base in the oligo-dT primer it is possible to use this procedure to amplify most of the polyA$^+$-containing mRNAs that are believed to be expressed in a typical cell at any one time.

The differential display method can be used to identify genes whose transcription is stimulated by contacting pancreatic cells with PDTF. RNA will be isolated from cells that have been so treated, and from identical cells which are not treated with PDTF. The RNA from each cell-type will then be reverse transcribed in four separate reactions using oligo-dT primers with dG, dA, dT or dC at the 3' terminus. The resulting cDNAs will be amplified in the presence of [^{35}S]dATP using a set of primers consisting of one of the four oligo-dT primers and one of 20 different commercially available primers of arbitrary sequence. Thus, for each tissue there will be 80 different PCR reactions, each involving the use of a different set of primers. The PCR products obtained using each set of primers will then be resolved on a denaturing polyacrylamide gel, with the PCR products resulting from the amplification of cDNA from the control target tissue run side-by-side with the PCR products resulting from the amplification of cDNA from the experimental target tissue. The PCR products will be visualized by autoradiography, and any products that are present in one lane but not the other will be cut out of the gel, subcloned and sequenced. Presumably these products represent the amplification of genes that are transcribed as a consequence of ectopic expression of the transcription factor.

Another aspect of the method of present invention provides a method for generating cultures of insulin-producing cells, by contacting a purified or semi-purified

preparation of a PDTF or cells expressing PDTF with a culture of pancreatic cells, pancreatic tissue or to a pancreatic explant as described below. Induction of growth which responds to the applied growth factor can be detected by proliferation of the progenitor cells.

In one embodiment, PDTF is contacted with small cross-sections of pancreatic ductal tissue under conditions sufficient to induce outgrowth of the tissue thereby forming new pancreatic tissue. In another embodiment, the PDTF can be contacted with micro-organ explant tissue, e.g., ductal tissue explants. Ductal tissue explants preferably are derived with dimensions that allow the explanted tissue to maintain its microarchitecture and biological function for prolonged periods of time in culture, e.g., the dimensions of the explant preserve the normal tissue architecture and at least a portion of the normal tissue function that is present in vivo. Such tissue explants can be maintained, for instance, in minimal culture media for extended periods of time (e.g., for 21 days or longer) and can be contacted with different factors, including PDTF. Carefully defined conditions can be acquired in the culture so as selectively activate discrete populations of cells in the tissue explant. Certain cells or tissues can be subsequently isolated from the explant, based upon its response to the addition of PDTF or other growth factors to the culture.

The micro-organ cultures used as substrates for PDTFs or PDTF-producing cells according to the invention preserve the normal tissue architecture that is present in vivo, e.g., the original epithelial-mesenchymal organization. In preferred embodiments, the populations of cells of the ductal explants are grouped in a manner that preserves the natural affinity of one cell to another, e.g., to preserve layers of different cells present in explant. Such an association facilitates intercellular communication. Many types of communication takes place among animal cells. This is particularly important in differentiating cells where induction is defined as the interaction between one (inducing) and another (responding) tissue or cell, as a result of which the responding cells undergo a change in the direction of differentiation. Moreover, inductive interactions occur in embryonic and adult cells and can act to establish and maintain morphogenetic patterns as well as induce differentiation (Gurdon (1992) Cell 68: 185-199). Exemplary micro-organ cultures which can be used in the method of the invention are described in the Examples and include epithelial and mesenchymal cells grouped in a manner that includes a plurality of layers so as to preserve the natural affinity and interaction of one cell to another in and between each layer.

In another embodiment, the ductal explants and/or pancreatic cells or tissue can be cultured on feeder layers, e.g., layers of feeder cells which secrete PDTF or polymeric layers containing PDTF. Natural or recombinantly engineered cells can be provided as feeder layers to the instant cultures.

Methods of measuring cell proliferation are well known in the art and most commonly include determining DNA synthesis characteristic of cell replication. There are numerous methods in the art for measuring DNA synthesis, any of which may be used according to the invention. In an embodiment of the invention, DNA synthesis has been determined using a radioactive label (^{3}H-thymidine) or labeled nucleotide analogues (BrdU) for detection by immunofluorescence.

In another preferred embodiment, the subject PDTF producing cells can be implanted into one of a number of regeneration models used in the art, e.g., partial pancreatectomy or streptozocin treatment of a host animal.

Accordingly, another aspect of the present invention pertains to the pancreatic tissue in which growth has been induced according to the invention.

Yet another aspect of the present invention concerns cellular compositions which include, as a cellular component, substantially pure preparations of the subject PDTF producing cells. Cellular compositions of the present invention include not only substantially pure populations of the PDTF producing cells, but can also include cell culture components, e.g., culture media including amino acids, metals, coenzyme factors, as well as small populations of non-PDTF producing cells, e.g., some of which may arise by subsequent differentiation of the cells of the invention. Furthermore, other non-cellular components include those which render the cellular component suitable for support under particular circumstances, e.g., implantation, e.g., continuous culture.

In one embodiment, the progenitor cells of the present invention are characterized by an ability for self-regeneration in a culture medium and differentiation to pancreatic lineages. In a preferred embodiment, the progenitor cells are inducible to differentiate into pancreatic islet cells, e.g., β islet cells, α islet cells, δ islet cells, or ϕ islet cells. Such pancreatic progenitor cells may be characterized in certain circumstances by the expression of one or more of: homeodomain type transcription factors such as PDX-1 (STF-1); PAX gene(s) such as PAX6; PTF-1; hXBP-1; HNF genes(s); villin; tyrosine hydroxylase; insulin; glucagon; and/or neuropeptide Y. The pancreatic progenitor cells of the present invention may also be characterized by binding to lectin(s), and preferably to a plant lectin, and more preferably to peanut agglutinin.

In yet another embodiment, the invention features a pharmaceutical composition including as the cellular component, a substantially pure population of PDTF producing cells, which cells are capable of inducing outgrowth of pancreatic tissue in a culture medium.

In general, the pancreatic ductal cells in which outgrowth is induced by PDTF or PDTF-producing cells will be of mammalian origin, e.g., cells isolated from a primate

such as a human, from a miniature swine, or from a transgenic mammal, or are the cell culture progeny of such cells. In one embodiment, pancreatic ductal tissue is isolated from a patient and subjected to the present method in order to provide a resulting culture of pancreatic cells or tissue (or differentiated cells derived therefrom). The isolated cells or tissue then can be transplanted back into the initial donor patient or into a second host patient.

In another aspect, the invention features, a method for screening a compound for ability to modulate one of growth, proliferation, and/or differentiation of progenitor cells obtained by the subject method, including: (i) establishing an isolated population of pancreatic progenitor cells; (ii) contacting the population of cells with a test compound; and (iii) detecting one of growth, proliferation, and/or differentiation of the progenitor cells in the population, wherein a statistically significant change in the extent of one of growth, proliferation, and/or differentiation in the presence of the test compound relative to the extent of one of growth, proliferation, and/or differentiation in the absence of the test compound indicates the ability of the test compound to modulate one of the growth, proliferation, and/or differentiation.

In another aspect, the invention features, a method for treating a disorder characterized by insufficient insulin activity, in a subject, including introducing into the subject a pharmaceutical composition including pancreatic cells derived by the subject method, or differentiated cells arising therefrom, and a pharmaceutically acceptable carrier.

In a preferred embodiment the subject is a mammal, e.g., a primate, e.g., a human.

In another preferred embodiment the disorder is an insulin dependent diabetes, e.g., type I diabetes.

In yet another preferred embodiment, the pancreatic duct cells are induced to differentiate into pancreatic islet cells, e.g., β islet cells, α islet cells, δ islet cells, or ϕ islet cells, subsequent to being introduced into the subject. Preferably, the pancreatic progenitors cells are induced to differentiate into pancreatic islet, e.g., β islet cells, α islet cells, δ islet cells, or ϕ islet cells, in culture prior to introduction into the subject.

The present invention also provides substantially pure progenitor cells which can be used therapeutically for treatment of various disorders associated with insufficient functioning of the pancreas.

To illustrate, the subject progenitor cells can be used in the treatment of a variety of pancreatic disorders, both exocrine and endocrine. For instance, the progenitor cells can be used to produce populations of differentiated pancreatic cells for repair subsequent to partial pancreatectomy, e.g., excision of a portion of the pancreas. Likewise, such cell

populations can be used to regenerate or replace pancreatic tissue loss due to, pancreatolysis, e.g., destruction of pancreatic tissue, such as pancreatitis, e.g., a condition due to autolysis of pancreatic tissue caused by escape of enzymes into the substance.

In an exemplary embodiment, the subject progenitor cells can be provided for patients suffering from any insulin-deficiency disorder. For instance, each year, over 728,000 new cases of diabetes are diagnosed and 150,000 Americans die from the disease and its complications; the total yearly cost in the United States is over 20 billion dollars (Langer et al. (1993) *Science* 260:920-926). Diabetes is characterized by pancreatic islet destruction or dysfunction leading to loss of glucose control. Diabetes mellitus is a metabolic disorder defined by the presence of chronically elevated levels of blood glucose (hyperglycemia). Insulin-dependent (Type 1) diabetes mellitus ("IDDM") results from an autoimmune-mediated destruction of the pancreatic β-cells with consequent loss of insulin production, which results in hyperglycemia. Type 1 diabetics require insulin replacement therapy to ensure survival. Non-insulin-dependent (Type 2) diabetes mellitus ("NIDDM") is initially characterized by hyperglycemia in the presence of higher-than-normal levels of plasma insulin (hyperinsulinemia). In Type 2 diabetes, tissue processes which control carbohydrate metabolism are believed to have decreased sensitivity to insulin. Progression of the Type 2 diabetic state is associated with increasing concentrations of blood glucose, and coupled with a relative decrease in the rate of glucose-induced insulin secretion.

The primary aim of treatment in both forms of diabetes mellitus is the same, namely, the reduction of blood glucose levels to as near normal as possible. Treatment of Type 1 diabetes involves administration of replacement doses of insulin. In contrast, treatment of Type 2 diabetes frequently does not require administration of insulin. For example, initial therapy of Type 2 diabetes may be based on diet and lifestyle changes augmented by therapy with oral hypoglycemic agents such as sulfonylurea. Insulin therapy may be required, however, especially in the later stages of the disease, to produce control of hyperglycemia in an attempt to minimize complications of the disease, which may arise from islet exhaustion.

More recently, tissue-engineering approaches to treatment have focused on transplanting healthy pancreatic islets, usually encapsulated in a membrane to avoid immune rejection. Three general approaches have been tested in animal models. In the first, a tubular membrane is coiled in a housing that contained islets. The membrane is connected to a polymer graph that in turn connects the device to blood vessels. By manipulation of the membrane permeability, so as to allow free diffusion of glucose and insulin back and forth through the membrane, yet block passage of antibodies and lymphocytes, normoglycemia was maintained in pancreatectomized animals treated with this device (Sullivan et al. (1991) *Science* 252:718).

In a second approach, hollow fibers containing islet cells were immobilized in the polysaccharide alginate. When the device was place intraperitoneally in diabetic animals, blood glucose levels were lowered and good tissue compatibility was observed (Lacey et al. (1991) *Science* 254:1782).

Finally, islets have been placed in microcapsules composed of alginate or polyacrylates. In some cases, animals treated with these microcapsules maintained normoglycemia for over two years (Lim et al. (1980) *Science* 210:908; O'Shea et al. (1984) *Biochim. Biochys. Acta.* 840:133; Sugamori et al. (1989) *Trans. Am. Soc. Artif. Intern. Organs* 35:791; Levesque et al. (1992) *Endocrinology* 130:644; and Lim et al. (1992) *Transplantation* 53:1180). However, all of these transplantation strategies require a large, reliable source of donor islets.

The pancreatic progenitor cells of the invention can be used for treatment of diabetes because they have the ability to differentiate into cells of pancreatic lineage, e.g., β islet cells. The progenitor cells of the invention can be cultured *in vitro* under conditions which can further induce these cells to differentiate into mature pancreatic cells, or they can undergo differentiation in vivo once introduced into a subject. Many methods for encapsulating cells are known in the art. For example, a source of β islet cells producing insulin is encapsulated in implantable hollow fibers. Such fibers can be pre-spun and subsequently loaded with the β islet cells (Aebischer et al. U.S. Patent No. 4,892,538; Aebischer et al. U.S. Patent No. 5,106,627; Hoffman et al. (1990) *Expt. Neurobiol.* 110:39-44; Jaeger et al. (1990) *Prog. Brain Res.* 82:41-46; and Aebischer et al. (1991) *J. Biomech. Eng.* 113:178-183), or can be co-extruded with a polymer which acts to form a polymeric coat about the β islet cells (Lim U.S. Patent No. 4,391,909; Sefton U.S. Patent No. 4,353,888; Sugamori et al. (1989) *Trans. Am. Artif. Intern. Organs* 35:791-799; Sefton et al. (1987) *Biotechnol. Bioeng.* 29:1135-1143; and Aebischer et al. (1991) *Biomaterials* 12:50-55).

Moreover, in addition to providing a source of implantable cells, either in the form of the progenitor cell population of the differentiated progeny thereof, the subject cells can be used to produce cultures of pancreatic cells for production and purification of secreted factors. For instance, cultured cells can be provided as a source of insulin. Likewise, exocrine cultures can be provided as a source for pancreatin.

As common methods of administering the cells of the present invention to subjects, particularly human subjects, which are described in detail herein, include injection or implantation of the cells into target sites in the subjects, the cells of the invention can be

inserted into a delivery device which facilitates introduction by, injection or implantation, of the cells into the subjects. Such delivery devices include tubes, e.g., catheters, for injecting cells and fluids into the body of a recipient subject. In a preferred embodiment, the tubes additionally have a needle, e.g., a syringe, through which the cells of the invention can be introduced into the subject at a desired location. The PDTF producing cells of the invention can be inserted into such a delivery device, e.g., a syringe, in different forms. For example, the cells can be suspended in a solution or embedded in a support matrix when contained in such a delivery device. As used herein, the term "solution" includes a pharmaceutically acceptable carrier or diluent in which the cells of the invention remain viable. Pharmaceutically acceptable carriers and diluents include saline, aqueous buffer solutions, solvents and/or dispersion media. The use of such carriers and diluents is well known in the art. The solution is preferably sterile and fluid to the extent that easy syringability exists. Preferably, the solution is stable under the conditions of manufacture and storage and preserved against the contaminating action of microorganisms such as bacteria and fungi through the use of, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. Solutions of the invention can be prepared by incorporating progenitor cells as described herein in a pharmaceutically acceptable carrier or diluent and, as required, other ingredients enumerated above, followed by filtered sterilization.

Support matrices in which the cells can be incorporated or embedded include matrices which are recipient-compatible and which degrade into products which are not harmful to the recipient. Natural and/or synthetic biodegradable matrices are examples of such matrices. Natural biodegradable matrices include plasma clots, e.g., derived from a mammal, and collagen matrices. Synthetic biodegradable matrices include synthetic polymers such as polyanhydrides, polyorthoesters, and polylactic acid. Other examples of synthetic polymers and methods of incorporating or embedding cells into these matrices are known in the art. See e.g., U.S. Patent No. 4,298,002 and U.S. Patent No. 5,308,701. These matrices provide support and protection for the fragile progenitor cells in vivo and are, therefore, the preferred form in which the progenitor cells are introduced into the recipient subjects.

The present invention also provides substantially pure embryonic pancreatic PDTF producing cells which can be used therapeutically for treatment of various disorders associated with insufficient functioning of the pancreas or liver. The currently preferred embryonic cells are murine e14.5 pancreatic mesenchymal cells. For example, the subject PDTF producing cells can be used in the treatment of a variety of pancreatic disorders, both exocrine and endocrine. For instance, the cells can be used to produce populations of pancreatic tissue or differentiated pancreatic cells for repair subsequent to partial pancreatectomy, e.g., excision of a portion of the pancreas. Likewise, such cell

populations can be used to regenerate or replace pancreatic tissue loss due to, pancreatolysis, e.g., destruction of pancreatic tissue, such as pancreatitis, e.g., a condition due to autolysis of pancreatic tissue caused by escape of enzymes into the substance.

In an exemplary embodiment, PDTF or PDTF-producing cells can be used to induce tissue growth in pancreatic tissue of a patient suffering from any insulin-deficiency. The tissue then can be activated using PYY, for example, and the activated tissue then can be implanted in the donor patient. Each year, over 728,000 new cases of diabetes are diagnosed and 150,000 Americans die from the disease and its complications; the total yearly cost in the United States is over 20 billion dollars (Langer et al. (1993) Science 260:920-926). Diabetes is characterized by pancreatic islet destruction or dysfunction leading to loss of glucose control. Diabetes mellitus is a metabolic disorder defined by the presence of chronically elevated levels of blood glucose (hyperglycemia). Insulin-dependent (Type 1) diabetes mellitus ("IDDM") results from an autoimmune-mediated destruction of the pancreatic β-cells with consequent loss of insulin production, which results in hyperglycemia. Type 1 diabetics require insulin replacement therapy to ensure survival. Non-insulin-dependent (Type 2) diabetes mellitus ("NIDDM") is initially characterized by hyperglycemia in the presence of higher-than-normal levels of plasma insulin (hyperinsulinemia). In Type 2 diabetes, tissue processes which control carbohydrate metabolism are believed to have decreased sensitivity to insulin. Progression of the Type 2 diabetic state is associated with increasing concentrations of blood glucose, and coupled with a relative decrease in the rate of glucose-induced insulin secretion.

The primary aim of treatment in both forms of diabetes mellitus is the same, namely, the reduction of blood glucose levels to as near normal as possible. Treatment of Type 1 diabetes involves administration of replacement doses of insulin. In contrast, treatment of Type 2 diabetes frequently does not require administration of insulin. For example, initial therapy of Type 2 diabetes may be based on diet and lifestyle changes augmented by therapy with oral hypoglycemic agents such as sulfonylurea. Insulin therapy may be required, however, especially in the later stages of the disease, to produce control of hyperglycemia in an attempt to minimize complications of the disease, which may arise from islet exhaustion.

More recently, tissue-engineering approaches to treatment have focused on transplanting healthy pancreatic islets, usually encapsulated in a membrane to avoid immune rejection. Three general approaches have been tested in animal models. In the first, a tubular membrane is coiled in a housing that contained islets. The membrane is connected to a polymer graph that in turn connects the device to blood vessels. By manipulation of the membrane permeability, so as to allow free diffusion of glucose and insulin back and forth through the membrane, yet block passage of antibodies and

lymphocytes, normoglycemia was maintained in pancreatectomized animals treated with this device (Sullivan et al. (1991) Science 252:718).

In a second approach, hollow fibers containing islet cells were immobilized in the polysaccharide alginate. When the device was place intraperitoneally in diabetic animals, blood glucose levels were lowered and good tissue compatibility was observed (Lacey et al. (1991) Science 254:1782).

Finally, islets have been placed in microcapsules composed of alginate or polyacrylates. In some cases, animals treated with these microcapsules maintained normoglycemia for over two years (Lim et al. (1980) Science 210:908; O'Shea et al. (1984) Biochim. Biochys. Acta. 840:133; Sugamori et al. (1989) Trans. Am. Soc. Artif. Intern. Organs 35:791; Levesque et al. (1992) Endocrinology 130:644; and Lim et al. (1992) Transplantation 53:1180). However, all of these transplantation strategies require a large, reliable source of donor islets.

PDTF or PDTF producing cells of the invention can be used for treatment of diabetes because they have the ability to induce growth of cells of pancreatic lineage, e.g., β islet cells. The PDTF producing cells of the invention can be cultured in vitro and used to induce pancreatic cells, such as duct tissue cells, to differentiate into mature pancreatic cells, or they can undergo differentiation in vivo once introduced into a subject. Many methods for encapsulating cells are known in the art. For example, a source of β islet cells producing insulin is encapsulated in implantable hollow fibers. Such fibers can be pre-spun and subsequently loaded with the β islet cells (Aebischer et al. U.S. Patent No. 4,892,538; Aebischer et al. U.S. Patent No. 5,106,627; Hoffman et al. (1990) Expt. Neurobiol. 110:39-44; Jaeger et al. (1990) Prog. Brain Res. 82:41-46; and Aebischer et al. (1991) J. Biomech. Eng. 113:178-183), or can be co-extruded with a polymer which acts to form a polymeric coat about the β islet cells (Lim U.S. Patent No. 4,391,909; Sefton U.S. Patent No. 4,353,888; Sugamori et al. (1989) Trans. Am. Artif. Intern. Organs 35:791-799; Sefton et al. (1987) Biotechnol. Bioeng. 29:1135-1143; and Aebischer et al. (1991) Biomaterials 12:50-55).

Moreover, in addition to providing a source of implantable cells, either in the form of a cell population or the differentiated progeny thereof, the subject cells can be used to produce cultures of pancreatic cells for production and purification of secreted PDTF and other factors. For instance, cultured cells can be provided as a source of insulin. Likewise, exocrine cultures can be provided as a source for pancreatin.

Yet another aspect of the present invention provides methods for screening various compounds for their ability to modulate growth, proliferation or differentiation of distinct progenitor cell populations from pancreatic duct tissue. A micro-organ explant that closely mimics the properties of a given set of tissue in vivo would have utility in

screening assays in which compounds could be tested for their ability to modulate one of growth, proliferation or differentiation of progenitor cells in such tissue. Requirements of a reproducible model for screening might include consistency in the micro-architecture, e.g. epithelial-mesenchymal interactions, and nutritional environment in vitro, as well as prolonged viability and proliferation of cultures beyond 24 hours to observe threshold effects of compounds being screened. This level of consistency cannot be achieved in the presence of undefined media supplements such as sera or tissue extracts that vary between batches and cannot be adequately controlled. The dependence of a model on external growth supplements such as growth factors is also undesirable as growth factors or hormones may be included among the compounds to be tested.

In an illustrative embodiment, ductal explants which maintain their microarchitecture in culture, e.g., they preserve the normal epithelial-mesenchymal architecture that is present in vivo, can be used to screen various compounds or natural products. Such explants can be maintained in minimal culture media for extended periods of time (e.g., for 21 days or longer) and can be contacted with any compound, e.g., small molecule or natural product, e.g., PDTF or other growth factor, to determine the effect of such compound on one of cellular growth, proliferation or differentiation of cells in the explant. Detection and quantification of growth, proliferation or differentiation of these cells in response to a given compound provides a means for determining the compound's efficacy at inducing one of the growth, proliferation or differentiation in a given ductal explant. Methods of measuring cell proliferation are well known in the art and most commonly include determining DNA synthesis characteristic of cell replication. There are numerous methods in the art for measuring DNA synthesis, any of which may be used according to the invention. In an embodiment of the invention, DNA synthesis has been determined using a radioactive label (^{3}H-thymidine) or labeled nucleotide analogues (BrdU) for detection by immunofluorescence. The efficacy of the compound can be assessed by generating dose response curves from data obtained using various concentrations of the compound. A control assay can also be performed to provide a baseline for comparison. Identification of the progenitor cell population(s) amplified in response to a given test agent can be carried out according to such phenotyping as described above.

In general, pancreatic stem cells which can be treated *ex vivo* with PDTF can be cultured, both before and after treatment with PDTF, by standard protocols known in the art. For instance, the cells can be cultured in defined or serum-derived media alone, or as co-cultures with a feeder cell layer, e.g., a stromal cell co-culture system. The isolated stem cells can be cultured in minimal essential medium supplemented with serum and antibiotics. Culture media that can be used include, for example, Hanks. McCoys, RPMI 1640 minimal essential media (MEM), and others, and include 1% to 20% serum.

Extracellular factors and cytokines that can de added are described below in the section *Expansion and Differentiation of Stem Cells*. While the subject stem cells can be grown in complex media, it will generally be preferred that the cells be maintained in a simple medium, such as Dulbecco's Minimal Essential Media (DMEM), in order to effect more precise control over the activation of certain progenitor populations in the culture.

The cells may be maintained in any suitable culture vessel, such as a 12 or 24 well microplate, and may be maintained under typical culture conditions for cells isolated from the same animal, e.g., such as 37°C in 5% CO_2.

In yet other embodiments, the stem cells can be cultured on feeder layers, e.g., layers of feeder cells which naturally, or by recombinant engineering, secrete PDTF, or polymeric layers containing PDTF factors. In preferred embodiments, the cells are expanded in a stromal cell co-culture system. See, for example, Deryugina et al. (1993) *Crit Rev Immunology* 13:115-150. Stromal cells are believed to provide not only a physical matrix on which stem cells reside, but also to produce membrane-contact signals and/or other pancreatic growth factors which can augment stem cell proliferation and differentiation. A variety of different stromal culture systems are available. In general, the modified stem cells are cultured on the stromal layer, and non-adherent cells isolated for further passage, implantation or other use.

Moreover, a variety of mammalian stromal cell lines are available and can be used to generate a confluent cell layer upon which the instant stem cell preparations can be cultured. Exemplary human stromal cell lines include KM-102, SV-MSC, ST-1, SCL and H-7 cell lines (see, for example, Deryugina et al., *supra*).

In another embodiment, a matrigel layer or the like can be used to induce expansion of the stem cell population. Matrigel (Collaborative Research, Inc., Bedford, Mass.) is a complex mixture of matrix and associated materials derived as an extract of murine basement membrane proteins, consisting predominantly of laminin, collagen IV, heparin sulfate proteoglycan, and nidogen and entactin was prepared from the EHS tumor as described Kleinman et al, "Basement Membrane Complexes with Biological Activity", Biochemistry, Vol. 25 (1986), pages 312-318. Other such matrixes can be provided, such as Humatrix.

Still other culture systems include the three-dimensional stromal cell and tissue culture system of the Slivka et al. U.S. patent 5,478,739 and the like.

In still another embodiment, the PDTF-treated cells of the present invention may be used *in vitro* to screen a wide variety of compounds, such as cytotoxic compounds, growth/regulatory factors, pharmaceutical agents, etc. To this end, the cultures are maintained *in vitro* and exposed to the compound to be tested. The activity of a cytotoxic

compound can be measured by its ability to damage or kill cells in culture. This may readily be assessed by vital staining techniques. The effect of growth/regulatory factors may be assessed by analyzing the cellular content of the culture, e.g., by total cell counts, and differential cell counts. This may be accomplished using standard cytological and/or histological techniques including the use of immunocytochemical techniques employing antibodies that define type-specific cellular antigens. The effect of various drugs on the cells may be assessed. For example, drugs that increase insulin productions can be tested.

Exemplification

The invention now being generally described, it will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Method for Generating Conditioned Media from the e14.5 Pancreatic Mesenchymal Cell Line

Establishing the Cell Culture

The culture was established from the an Immorto-Mouse strain. Briefly, the fetal pancreas from the Immorto-Mouse was isolated at day 14.5 of gestation. After a collagenase/dispase treatment the cell suspension was filtered through a 0.7 micron filter and plated for growth. Initially the culture was maintained in a transwell over a feeder of LIF-producing cells. Subsequently, the cells were plated and maintained on platic, first with and then without LIF-conditioned media. The culture is heterogeneous, containing at least two different cell types: Large mesenchymal cells and small epithelial-like cells. The cell line was further characterized for its ability to stimulate outgrowth of cells from pancreatic duct slices.

Generating Conditioned Media

Standardly, cells were maintained in complete medium [DMEM basal medium with 10% Fetal Bovine Serum (FBS) and gamma-Interferon (g-IFN)], at 33°C and 5% CO2.

For generating conditioned media (CM), cells were grown to (sub)confluency at which time the culture was switched to serum-free medium [SFM=DMEM basal medium with/without 0.05% Bovine Serum Albumin (BSA)]. Adding BSA did not affect the "activity" of the CM. Cell were further incubated at 37°C and 5% CO2.

The CM was harvested every 24 hrs for 3-4 days, from each cell monolayer. After pooling of the samples, the CM was concentrated 10-20 fold with the aid of a Amicon Concentrator: Under the pressure of liquid nitrogen the CM was forced through a filter with a size cut-off of 10K (KiloDalton).

The concentration CM was subsequently used for the Duct Outgrowth and Proliferation assays described below.

CM fractionation

Serum-free CM was collected from monolayer cultures of e14.5 pancreatic mesenchymal cells and filtered through 0.45um filter unit (by Oivin). CM was concentrated about 100-fold using the Amicon concentrator and YM 10 membrane, the concentrate was injected onto a FPLC Superose 12 column, equilibrated, and eluted with phosphate buffered saline (PBS) containing 0.5 M NaCl. The first 7 ml eluted was discarded, and then 0.5-ml fractions were collected.

Sample Preparation for Bioassay

Aliquots from fractions were treated as follows. One hundred microliters of 10% fetal bovine serium/DMEM (as carrier) and 2 ml of DMEM were added to each sample, and the sample was concentrated to 0.2 ml on a Cnetricon-10 concentrator (Amicon). The retentate was brought back to 2 ml with DMEM and reconcentrated twice. The sample was sterilized via Spin-X unit (pore size, 0.45um; Costar) and then added to the culture at day 0.

Preparation of Pancreatic Ductal Explants Cultured with Fractionated e14.5 Pancreatic Mesenchyme Conditioned Medium

The pancreatic ducts were dissected out from 6 week old female Sprague/Dawley rats and transferred to cold Hanks Buffer (P/S) on ice. One duct at a time was transferred to cold Iscoves Medium (P/S) and pancreatic and fat tissue were cleaned off. After the ducts are cleaned of fat and pancreatic tissue, they were transferred to fresh Iscoves medium, and allowed to sit on ice for 10 minutes. The cold ducts then were sectioned at 200μm per section. The sections were carefully transferred to fresh cold Iscoves in a 35mm dish an allowed to sit on ice for 20 minutes. While the ducts were sitting on ice, 300μl of matrigel-reduced was added to a 12-well plate and allowed to solidify at room temperature for 15 minutes. Duct sections then were placed in the wells, 10 per well, and allowed to stand at room temp for 30 minutes to allow the ducts to adhere to the matrigel.

Conditioned medium prepared as described above was added to appropriate wells, and incubated at 37°C with 5% CO_2. The cultures were fed with fresh medium and e14.5PM conditioned medium every 3-4 days and monitored for growth. The cultures were terminated after two weeks.

Results:

After two weeks in culture, the ducts treated with fractions B, E, F, and G had significant growth. Growth was compared to the controls with e14.5 total conditioned medium and DMEM. (Figures 9 and 10).

Treatment of Pancreatic Duct Tissue from Wildtype and PDX-1/LacZ Mice with e14.5 Conditioned Medium

Pancreatic ducts were dissected as described above from wildtype and PDX-1/LacZ adult mice. The ducts were cleaned of fat and pancreatic tissue. The ducts were sectioned by hand as described above. Ten ducts were placed into 12-well plate coated with Matrigel-growth factor reduced. The cultures were fed with fresh medium and conditioned medium every 3-4 days and monitored for growth. The cultures were terminated after two weeks.

Results:

In the wildtype mouse cultures the ducts cultured with fractions D, E, and F had the most noted growth compared to the controls with e14.5 total conditioned medium and DMEM. (Figure 11)

The PDX-1/LacZ mouse duct explants demonstrated growth with most of the fractions except fractions C, D, and K. (Figure 12) The cells were treated with protease to remove from matrigel then plated onto slides and stained for the presence of beta-galactosidase. B-gal expression was seen in cells cultured with fractions D, E, and F. (Figures 13 and 14)

Presentation of Ductal Fragment Cultures

The common bile duct (CBD) is a structure whose developmental origins is poorly understood. Its primary function is the delivery of bile acids from the liver to the duodenum to aid in the emulsification of food in the digestive process. 80% of its length traverses through the pancreas and is connected into the pancreatic ductal system through a series of anastamoses. Through these anastamoses and into the CBD flow the digestive enzymes secreted by the exocrine pancreas. The relation of the CBD to the liver and pancreas, and its morphology, are therefore essential to both liver and pancreas normal function.

Since it contributes to both pancreatic and hepatic function, the question arises as to whether the CBD arises during development as primarily a hepatic structure, a pancreatic structure, or both. Little has been done to resolve this issue in the past due in part to a lack of early markers as well as interest. However, there are a number of reports in the literature citing circumstantial evidence that the adult animal retains within the gut system stem cells for both liver and pancreas.

Because the CBD serves a dual hepato-pancreatic function, it might be possible that it also possesses a dual identity, and might then retain within its structure stem cells for both the pancreas and liver. To test this hypothesis directly we isolated the CBD and its attendant main ducts, and cultured them in vitro as intact duct segments. The goal of this work was to study the duct as an intact physiological unit and to determine whether resident stem cells could be activated to give rise to either liver and/or pancreatic derivatives. This unique culture system allows study of the interaction of the mesenchyme with the epithelium, rather than the isolated culture systems of others, and is hence more representative of the in vivo situation.

Using this unique culture system cultures of ductal fragments were derived from the common bile duct of the adult rat in a combination of matrigel and IGF-1 in serum

free conditions induced the formation of liver specific cell types. The liver identity of the induced cells was determined by immunohistochemical detection of the expression of alphafetoprotein (AFP), albumin, and ATBF-1, a transcription factor shown to regulate AFP expression. In addition, the formation of red blood cell clusters was observed. The induction of albumin and AFP positive cell types was specific for the matrigel/IGF-1 combination; culture on plastic or collagen with IGF-1 failed to induce the appearance of these cell types. Likewise, the substitution of IGF-1 with either TGFα, EGF, IGF-II, PDGF, or FGFβ, all failed to elicit liver formation, even in the presence of matrigel. However, in all of the matrigel conditions tried, we observed the formation of red blood cell clusters. Our results indicate that there exist both liver and pancreatic stem cells resident in the CBD system, that red blood cell formation can be stimulated by a factor present in matrigel, and that the combination of matrigel with IGF-1 can induce the formation of liver specific cell types.

Preparation of Ductal Explants

Six week old female Sprague/Dawley from Taconic were exsanguinated by CO_2. The common bile duct and associated pancreatic ducts was removed and placed in cold Dulbecco's Minimal Essential Medium (DMEM) supplemented with 2mM glutamine and penicillin/streptomycin (100units/ml). The duct was then cleaned of associated pancreatic tissue, liver tissue, fat, and blood vessels. The clean and intact duct was sectioned into approximately 250 um transverse slices, such that the original epithelial-mesenchymal microarchitecture was retained, and placed in medium on ice.

250µl of reduced growth factor Matrigel (Collaborative Research) was added to each well of a 24-well plate and incubated at 37°C for 30 minutes. The plates were then removed and allowed to stand at room temperature for 15 minutes. The CBD microorgan explants were placed onto the matrigel per well and stood at room temperature for 15 minutes to allow the ducts to adhere to the matrigel. 1 ml of DMEM (P/S/L-glut) with or without growth factor addition was added to the wells. Growth factors used in the studies described below (EGF, TGFα, PDGF, FGFβ, FGFα, IGF-1, and IGF-II) were all obtained from PreproTech. Cultures were incubated at 37°C in a 5% CO_2 atmosphere. Cultures were fed once a week with fresh medium.

Immunohistochemistry

The cultures were fixed overnight at 4°C in 4% paraformaldehyde (PFA). The cultures were rinsed three times 10 minutes each with phosphate-buffered saline (PBS) prior to addition of 1% periodic acid to remove endogenous peroxidase activity. The cultures were incubated with 3% milk in PBS/0.1% Triton X-100 (PBST) for 30 minutes

at room temperature. Primary antibody to alphafetoprotein and albumin (Accurate) were added at 1:250 for 1 hour at room temperature. ATBF-1 was used at 1:1000. The tissue was washed 3 times with PBST over a period of 2 hours. The secondary biotinylated antibody (Vector) was added at 1:500 for 1 hour at room temperature. The tissue was then washed 3 times for 1 hour with PBST and incubated with horseradish peroxidase-conjugated to avidin (Vector) for 30 minutes at room temperature. Antibody-binding was detected by the addition of DAB/H_2O_2 (Gibco). After termination of the colorimetric reaction with H_2O, 80% glycerol in PBS was added to clear the cultures prior to photograph.

Growth Factors

Effectiveness of the growth factors in stimulating proliferation was judged by the incorporation of Bromodeoxyuridine (BrdU) into DNA by the responding cells. Antibodies to BrdU were used to visualize and characterize the short term responses (24-48 hr).

The long term response was judged by the ability of these populations of cells to be grown and expanded in cell culture as a result of specific growth factor addition.

Different growth factors (EGF, TGF-α, bFGF, aFGF, IGF-I, IGF-II, VEGF and HGF) were used. In addition to the results for EGF, TGF-α and bFGF described below, IGF-I, IGF-II, VEGF and HGF were demonstrated to cause expansion of certain subpopulations of the ductal explant.

1. Administration of EGF to the CBD Explant

EGF was administered in three different doses, 1 ng/ml, 10 ng/ml and 100 ng/ml to the CBD explant. Activation of proliferation as assessed by BrdU labeling occurred with administration of 10 ng/ml of growth factor EGF within a span of 24 hr. There was no difference observed between 10 and 100 ng/ml dose. Addition of EGF to the CBD tissue explant resulted in proliferation of distinct cells within the explant and resulted in clustering of these cells.

2. Administration of TGFα to the CBD Explant

TGFα was administered in the same doses as EGF. Activation of proliferation as assessed by BrdU labeling occurred with administration of 100 ng/ml of growth factor TGF-α within a span of 24 hr. Unlike EGF, administration of TGF-α to the CBD explant resulted in proliferation of cells throughout the explant.

3. Administration of FGFβ to the CBD Explant

FGFβ was administered in the same doses as the above described growth factors. Activation of proliferation as assessed by BrdU labeling occurred with administration of 10 ng/ml of growth factor FGFβ within a span of 24 hr. Administration of 10 ng/ml of FGFβ resulted in induction of distinct CBD structures to synchronously divide, implying organized regulation of proliferative potential and response. Figure 1 depicts in vitro growth and expansion of CBD explant, e.g., formation of outgrowths, e.g., blebs, in response to administration of FGFβ.

Preliminary long term growth experiments indicate that there does exist a large proliferative potential within the CBD explant that can be maintained in culture for at least 21 days.

Characterization of Expanded Progenitor Cell Populations

Antibodies that recognize transcription factors known to be expressed during pancreas and liver formation were used to characterize the population distribution in CBDs. STF-1, also known as IPF-1, has been shown to be critical in pancrease formation; Pax-6 has been shown to be critical in pancreatic endocrine cells; Islet-1 is expressed in early gut endoderm and in islet tissue; and HNF3β is expressed in early gut endoderm and in liver precursor cells in the endoderm. ATBF-1, Lim1/2 were not detected in the CBD. Table 1 shows the average number of positive nuclei for each marker per 20X field, or approximately every 500μm duct length.

Table 1

Scoring of Marker Distribution in the CBD cryostat sections

Markers	# of nuclei	Average
STF-1	209 170 300	226
Pax-6	26 10 18	18
HNF3β	55 46 50	50
Islet-1	33 42 38	38

HNF3β is an early marker for endoderm formation and is one of the earliest markers expressed by the developing liver. Sections of CBD were stained for the early endoderm and liver marker HNF3β. The common bile duct (CBD) contains cells that are immunopositive for HNF3β. Its expression in the CBD is sporadic but specific to the epithelium, as expected. HNF3β is expressed throughout the CBD and also in the main pancreatic ducts, although less frequently. Positive cells were often found in clusters of epithelial cells that were branching away from the main lumen of the CBD. HNF3β-expressing cells were found uniformly throughout the CBD. Because of HNF3β's role as an early endoderm and liver marker in embryonic development, we hypothesized that these HNF3β expressing cells might be liver stem cells resident in the adult hepatopancreatic duct system.

If the observed HNF3β expressing cells are indeed liver precursors one might then use ductal cultures to attempt to in vitro activate the formation of liver-specific cell types and structures. Construction of such a culture system would then allow the study of the signals and interactions required to induce the liver development program. To this end we cultured duct fragments that had been transversely cut into approximate 250 μm widths.

These were then cultured either on plastic, collagen, directly on feeder layers (STO, C3H10T1/2), or on matrigel (Collaborative Research, Inc., Bedford, Mass.). Matrigel was the only matrix permutation tried that gave liver formation. Furthermore, the formation of liver structures only occurred with the addition of IGF-1. Addition of FGFβ, TGFα, EGF, IGF-II, PDGF-AA all failed to induce the formation of liver structures.

The addition of IGF-1 to duct cultures gave rise to three basic duct colonies; "Nonresponders" in which there was little observed growth or change in morphology, "N-type" colonies which underwent dramatic changes in morphology to give rise to neuritelike processes which were in fact fibroblasts laying end on end and "L-type" colonies which took on a liver-like morphology, with epithelial blebs and the formation of red blood cell clusters. This liver-like morphology resembles that of cultured embryonic liver (data not shown).

Because of the morphological similarity between our L-type colonies and cultured embryonic liver, we decided to look for the expression of liver-specific markers in all three colony types. The markers used for this analysis were alphafetoprotein (AFP), albumin, and ATBF-1. All three have been shown to be specific for early liver. In all cases examined the only cultures which stained immunopositive for all three markers were the L-type colonies.

For example, cultures were stained for the expression of the early liver marker AFP. AFP is expressed within 24 hours of liver formation in embryos, and is lost in adult hepatic tissue. Our data indicated extensive expression of AFP in IGF-1 treated duct fragments. We also saw loose cells that were highly positive for AFP. These tended to be large cells in diameter. We also observed the formation of duct-like structures that have arisen in the CBD explants. These duct-like structures were often surrounded by highly positive AFP staining cells. Some of the duct-like structures appear themselves to be AFP positive, probably due to the fact that AFP is a secreted factor.

Cultures were also stained for the early liver marker ATBF-1. ATBF-1 has been shown to regulate expression of AFP during embryonic liver development. We observed staining of L-type colonies with the liver marker ATBF-1.

In another line of experiments, progenitor cells from the ductal cultures were expanded by treatment with IGF-1, and the markers insulin, glucagon, amylase and PDX-1 were detected.

Briefly, cells were treated with protease after 2 weeks in culture to dissociate cells from matrigel. The cells were stained overnight at 4°C with primary antibody. The cells were then incubated one hour with secondary antibody at room temperature followed by a one hour incubation with either a FITC conjugate or a Cy3 conjugate to visualize. The staining conditions were as follows: 1. Insulin/Pax6; 2. Glucagon/Pax6; 3. PDX-1; 4. Amylase. 100 cells were counted and the number of positive cells for each marker was tabulated. Note that all of the Pax6 positive cells were insulin positive, but not the reverse. This was also the case for Glucagon/Pax6, where not all the Glucagon cells were Pax6 positive.

Table 2: Pancreatic marker staining on cells generated from ductal fragment outgrowths.

Markers		Pax6
Insulin	81 (81%)	26 (26%)
Glucagon	66 (66%)	10 (10%)
PDX-1	37 (37%)	
Amylase	26 (26%)	

The protocol for the experiments of Table 2 is as follows:

Directions: The common bile duct was dissected from 6 week old female Sprague/Dawley rats. The duct was cleaned of fat and pancreatic cells. The ducts were then sectioned in 200uM sections and transferred to Iscove's Medium with 5% Penicillin/Streptomycin (P/S) for 5 minutes, then transferred to Iscove's Medium with 1% P/S on ice.

Feeder cell lines: 14.5 pancreatic mesenchyme (PM) was split one day before the experimental set-up into 12 well transwell inserts. The cells were seeded at 2.5 X 10^4 cells/well. 0.5 ml. of DMEM with 1% PS/G, Beta-mercapthanol, and gamma interferon (50 units/ml) were added. The cells were incubated at 37 C with 5% CO_2.

Culturing conditions: The ductal fragments were placed in 12-well plates coated with 300µl matrigel-reduced growth factor (Collaborative Research). 1 ml of DMEM low glucose with 1% P/S/G was added. Transwells plated with 14.5 pancreatic mesenchyme were inserted into the wells with the ductal fragments. The cocultures were incubated at 37 C with 5% CO_2. The cultures were fed every 3-4 days with fresh medium and new transwells of 14.5 PM every 7 days. The cultures were terminated after 2 weeks and the ductal outgrowths were analyzed by immunohistochemistry.

Dissociation of cells: To dissociate the cells, protease was added for 30 minutes at 37 C. The cells were gently pipetted up and down and then transferred to an eppendorf. The cells were microfuged at 1800rpm for 1 minute. The cells were washed once with PBS. The cells were resuspended in PBS and cytospun onto slides. 1% PFA was used to fix the cells for 5 minutes at room temperature. The cells were washed 3 times for 5 minutes each with PBS. The slides were stored at 4°C.

Immunohistochemistry: The slides were washed once with PBS. 3% milk in PBS/0.1% Tween was used as the blocking agent. Primary antibody was made with 1% milk in PBS/0.1% Tween and incubated overnight at 4°C. The slides were washed 3 times, 20 minutes each with PBS/0.1% Tween. Secondary antibodies were added and incubated at room temp for one hour. The slides were washed 3 times with PBS/0.1% Tween. To visualize FITC conjugate or Cy3 avidin D was added for one hour at room temp. From 100 cells the number of positive cells were counted.

Injection of Conditioned Media into Mice

Figure 23 shows injection of 20X concentrated e14.5 PM cell line conditioned medium (CM) stimulates PDX-1 expression in pancreatic ductal epithelium of adult mice. Two days serum-free conditioned medium was concentrated to 20X using a Centricon filter with a 10kD cutoff range. Mice were injected with 200 ul of concentrated CM for 7 days followed by 3 days of no injection. Pancreata were collected, fixed, paraffin sectioned, and stained for PDX-1 expression. 2 of 3 mice injected with CM gave rise to extensive PDX-1 expression in the ductal epithelium. Staining was seen predominantly in the larger ducts including the common bile and pancreatic ducts and main duct. The interlobular ducts were not seen to contain as much PDX-1 expression.

In contrast, Figure 24 shows a representational section of a DMEM injected control mouse. No PDX-1 expression was detected in any of the 3 mice injected. Briefly, three mice were injected with DMEM identically as with CM as described above, and the pancretta analyzed for PDX-1 expression. Whereas 2/3 mice injected with CM gave rise to extensive PDX-1 staining in the ductal epithelium, all 3 control mice showed no evidence of inducible PDX-1 expression.

TGFβ proteins stimulate expansion/growth of pancreatic progenitor cells

TGFβ proteins are stable, mutlifunctional polypeptide growth factors. Proteins in this family have effects on growth and differentiation of many cell types. We tested the effects of TGFβ2, 3 and 5 on adult ductal explants to determine if these closely related growth factors (70-80% sequence homology) had any effects on growth and/or differentiation of pancreatic progenitor cells we have isolated from the explants.

Various feeder cell lines were tested to see if any would have an effect on expansion of outgrowth from the explant. The feeders that were assayed included FL1, FL2, FL3, CHO, Cos, 3T3, and 10T1/2 cells. FL3 in transwells and the corresponding conditioned medium had the most effect on progenitor cell outgrowth of the explants.

Immunohistochemical analysis on the cell outgrowth from FL3 conditioned medium have shown positive staining for insulin, glucagon, somatostatin and PDX-1.

In our efforts to elucidate the factor(s) that stimulate growth by the conditioned medium, the conditioned medium was fractoinated over a reverse-phase sizing column. These fractions were assayed in the rat explant assay and the intial results demonstrated that growth occurred in three out of the ten fractions. The peak of outgrowth activity revealed the size of the factor or factors to be in the approximate range of 29-44 kD.

In parallel, the conditioned medium was boiled for 5 minutes at 95°C, then assayed on the ductal explants. The conditioned medium mantained its simtulatory activity on progenitor cell outgrowth, indicating that the factor(s) is heat stable. See Figure 16.

Taken together, these data suggest that the factor in the conditioned medium might be a TGFβ protein. TGFβ1, TGFβ2, TGFβ3 and TGFβ5 were assayed; all were positive except TGFβ1. See Figures 17, 18 and 22. Moreover, the effects of the TGFβ2, TGFβ3 and TGFβ5 on expression of PDX-1 in the explant was also compared with the conditioned media. See Figures 19, 20 and 21.

Briefly, the main pancreatic duct was dissected out of an adult mouse or rat. The duct was cleaned of fat and pancreatic tissue. The duct was sectioned into 200μm sections and placed on ice. A 12 well or 24 well plate was coated with matrigel-reduced growth factor. The matrigel was allowed to solidify for 15 minutes at room temperature. The ducts were individually placed on top of the matrigel. To allow the duct to adhere to the matrigel, the plate was incubated at room temperature for 30 minutes. After 30 minutes, DMEM (low glucose) was added to each well. TGFβ protein, at appropriate concentrations, were added to the culture. The culture was incubated at 37°C with 5%CO_2. Every 3-4 days, spent media was removed and fresh media and TGFβ was added.

All of the above-cited references and publications are hereby incorporated by reference.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed is:

1. A pancreatic ductal trophic factor expressed by e14.5 pancreatic mesenchyme cells, wherein said factor is capable of inducing outgrowth of pancreatic ductal tissue.

2. A pancreatic ductal trophic factor composition characterized by a protein component capable of inducing outgrowth of pancreatic ductal tissue, which protein component

(i) has an apparent molecular weight in the range of 40-50kD,

(ii) is heat stable, and

(iii) is protease sensitive

3. A conditioned medium comprising at least one pancreatic ductal trophic factor expressed by e14.5 pancreatic mesenchyme cells.

4. The pancreatic ductal trophic factor of claim 1 or 2, which is a growth factor selected from a group consisting of IGF, EGF, TGF, FGF, HGF and VEGF, or orthologous or paralogous factors thereof.

5. The pancreatic ductal trophic factor of claim 1 or 2, which is a growth factor of the TGFβ superfamily.

6. The pancreatic ductal trophic factor of claim 5, which is a growth factor selected from the group consisting of TGFβ1, TGFβ2, TGFβ3, TGFβ4 and TGFβ5.

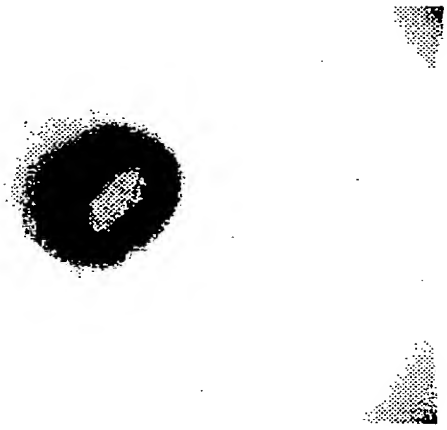
7. The pancreatic ductal trophic factor of claim 5, which is a growth factor has an amino acid sequence which is encodable by a nucleic acid which hybridizes to a TGFβ gene selected from the group consisting of TGFβ1, TGFβ2, TGFβ3, TGFβ4 and TGFβ5.

8. The pancreatic ductal trophic factor of claim 5, which is a growth factor has an amino acid sequence at least 70 percent identical to a TGFβ gene selected from the group consisting of TGFβ1, TGFβ2, TGFβ3, TGFβ4 and TGFβ5.

9. A pharmaceutical composition comprising the pancreatic ductal trophic factor of any of claims 4-8.

10. A method of inducing outgrowth of pancreatic duct tissue comprising contacting said duct tissue with a pancreatic duct trophic factor of any of claims 4-8.

11. A method of inducing outgrowth of pancreatic duct tissue comprising contacting said duct tissue with a conditioned medium obtained from a culture of e14.4 mesenchyme cells which express a pancreatic duct trophic factor.

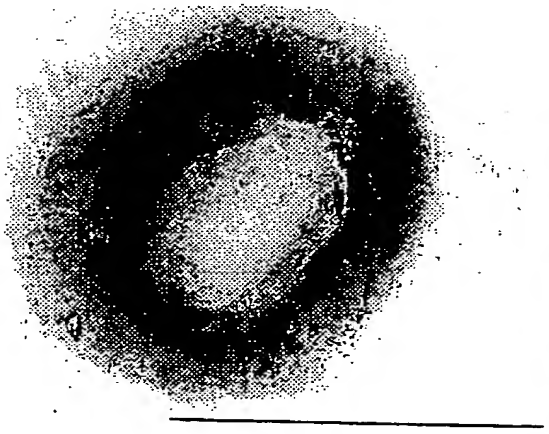


WildType (4X)

Fig. 1A

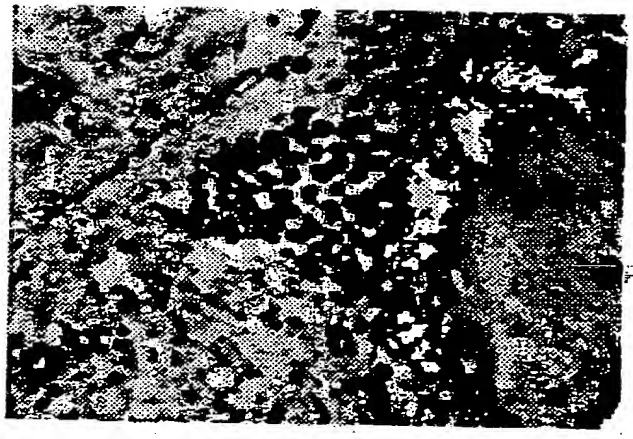
PDX-1/LacZ (4x)

Fig. 1B



WildType (20X)

Fig. 1C



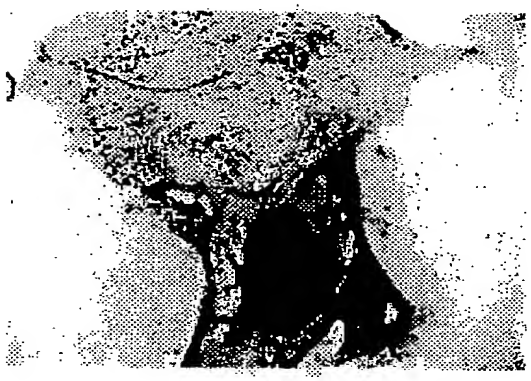
PDX-1/LacZ (20x)

Fig. 1D

Wild Type Ductal Explant + DMEM

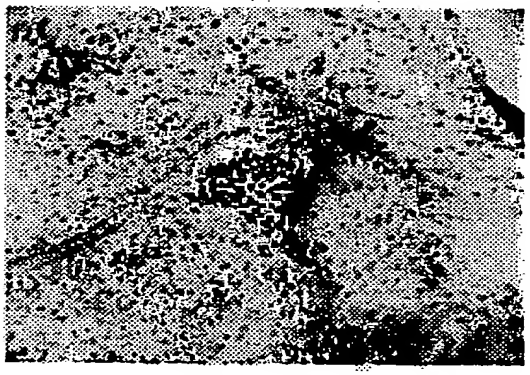
Fig. 2A

PDX-1/LacZ Ductal Explant + e14.5 PM



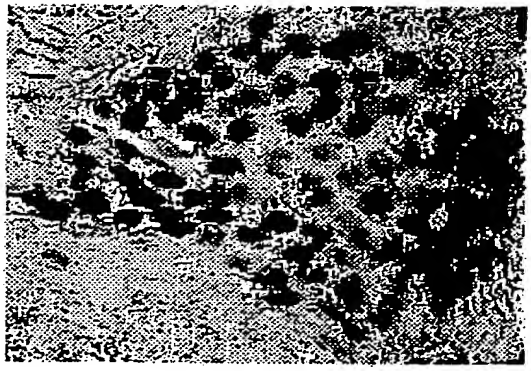
4X

Fig. 2B



10X

Fig. 2C



20X

Fig. 2D

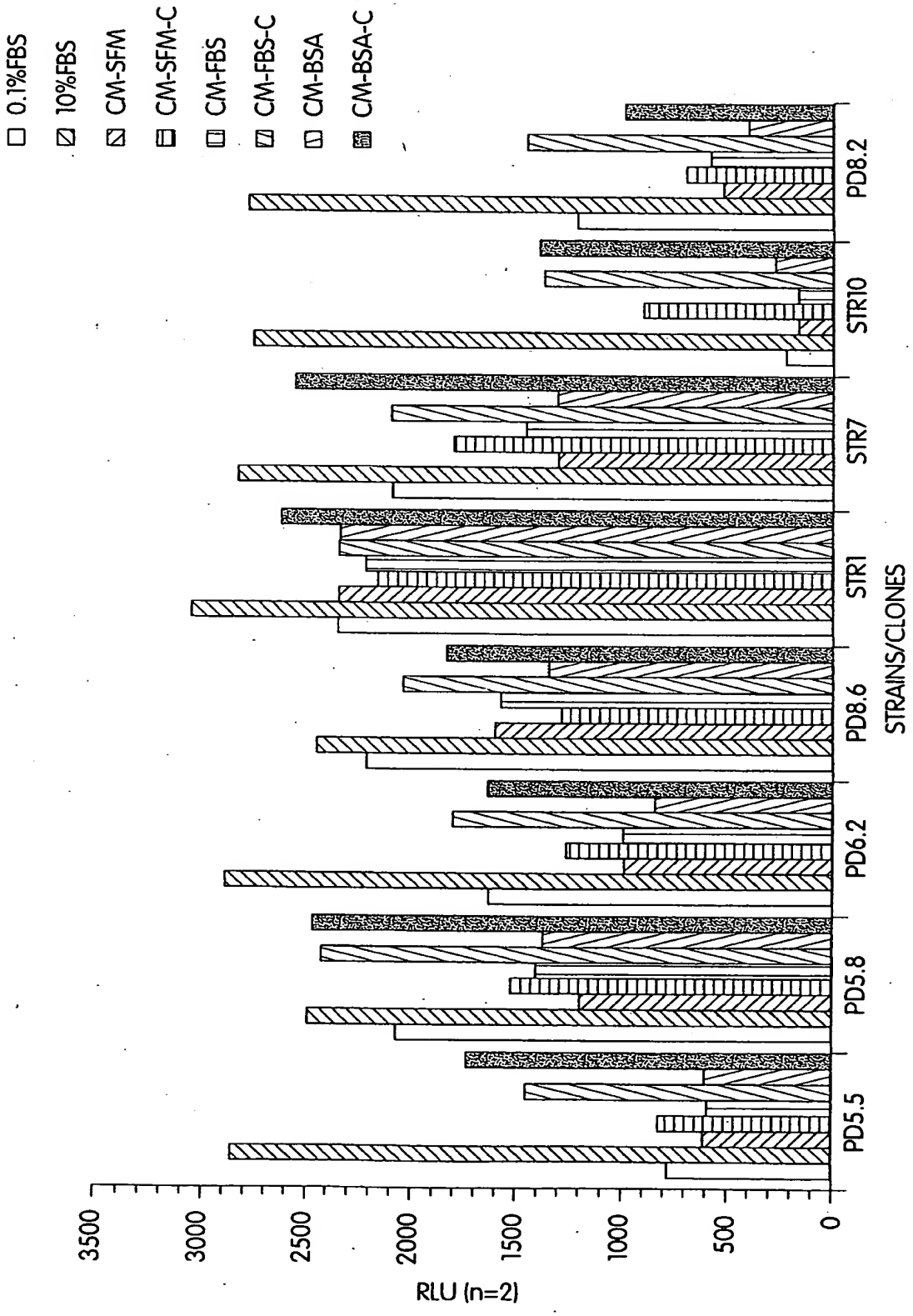

0.1%FBS
10%FBS
CM-SFM
CM-SFM-C
CM-FBS
CM-FBS-C
CM-BSA
CM-BSA-C
PD8.2
STR10
STR7
STR1
PD8.6
PD6.2
PD5.8
PD5.5
STRAINS/CLONES
3500
3000
2500
2000
1500
1000
500
0
RLU (n=2)

Fig. 3

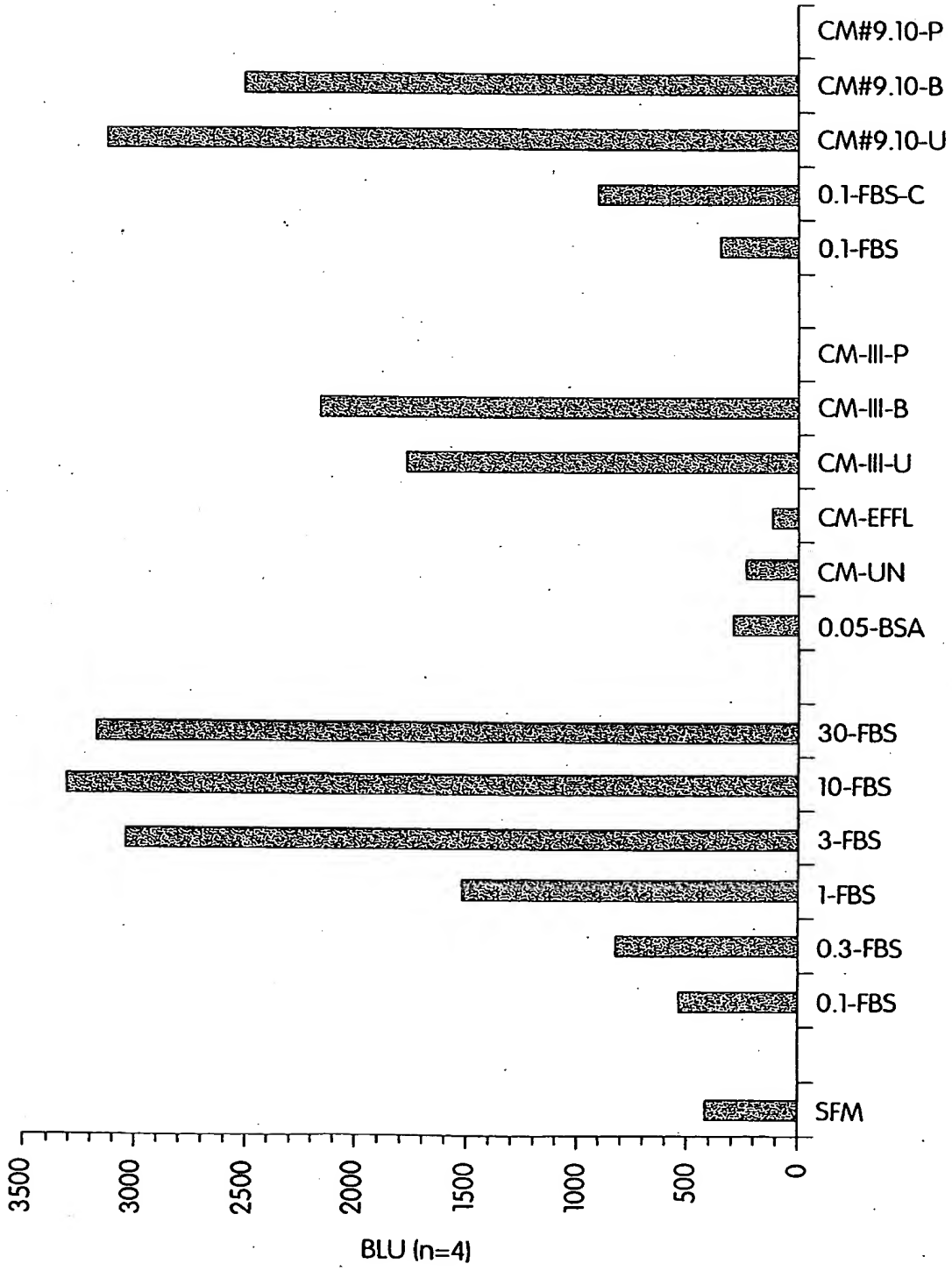

CM#9.10-P
CM#9.10-B
CM#9.10-U
0.1-FBS-C
0.1-FBS
CM-III-P
CM-III-B
CM-III-U
CM-EFFL
CM-UN
0.05-BSA
30-FBS
10-FBS
3-FBS
1-FBS
0.3-FBS
0.1-FBS
SFM
3500
3000
2500
2000
1500
1000
500
0
BLU (n=4)
GROWTH CONDITIONS

Fig. 4

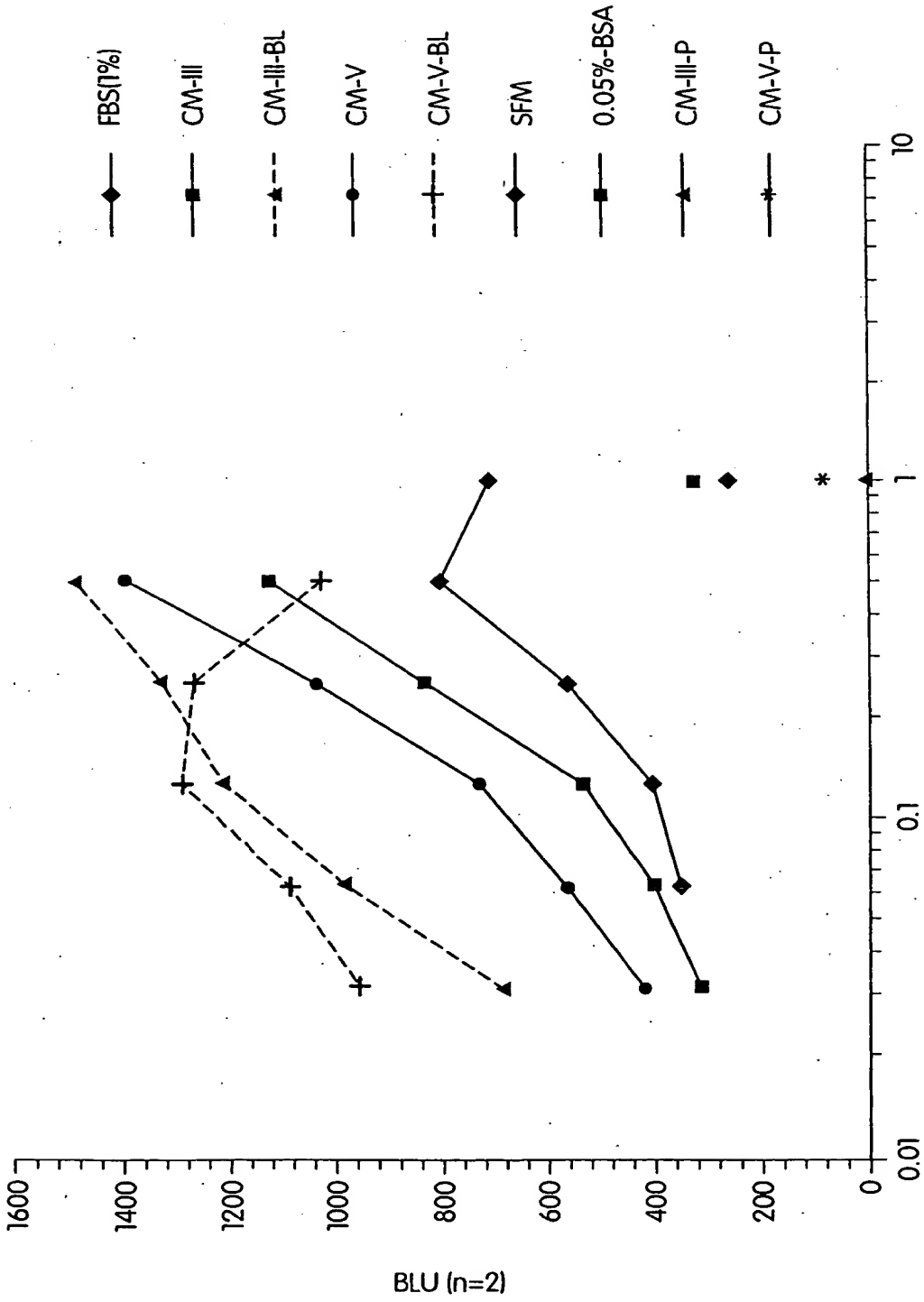
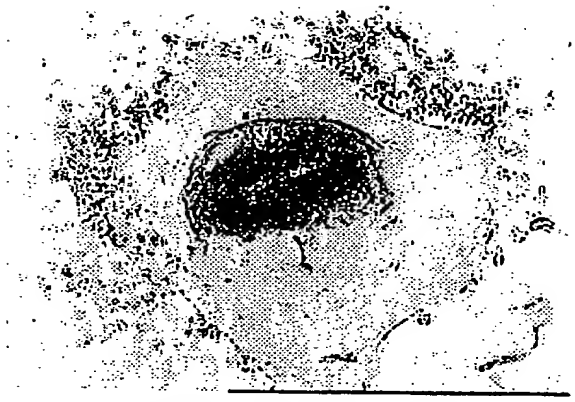
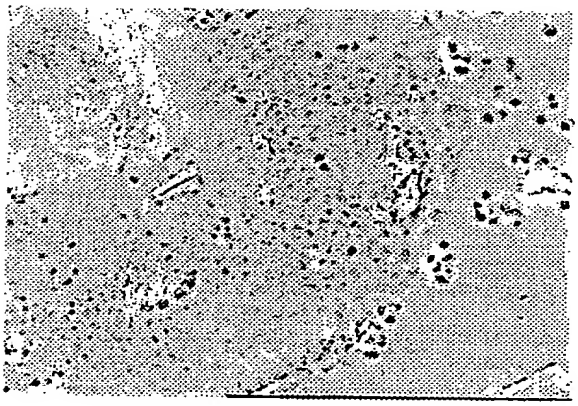

FBS(1%)
CM-III
CM-III-BL
CM-V
CM-V-BL
SFM
0.05%-BSA
CM-III-P
CM-V-P
1600
1400
1200
1000
800
600
400
200
0
0.01
0.1
1
10
DILUTIONS
BLU (n=2)

Fig. 5



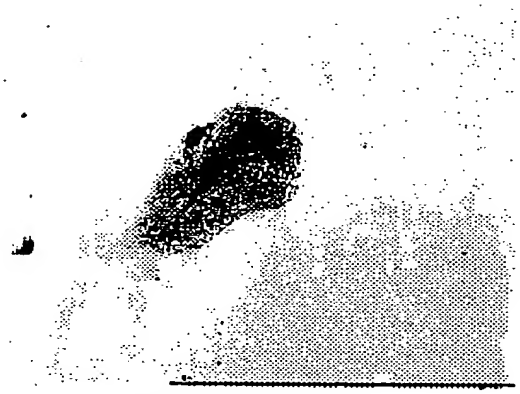
Conditioned Medium III (4x)

Fig. 6A



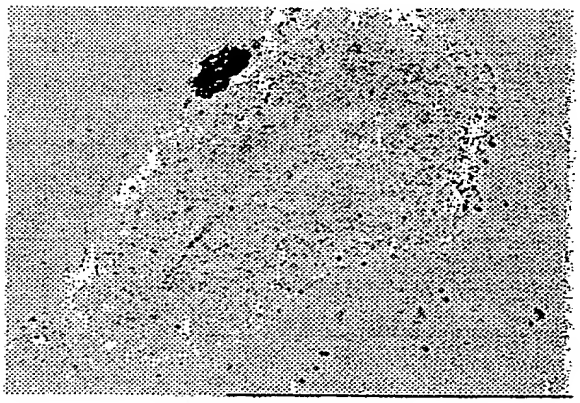
Conditioned Medium III (10x)

Fig. 6B



Flow through (4x)

Fig. 6C



Flow through (10x)

Fig. 6D

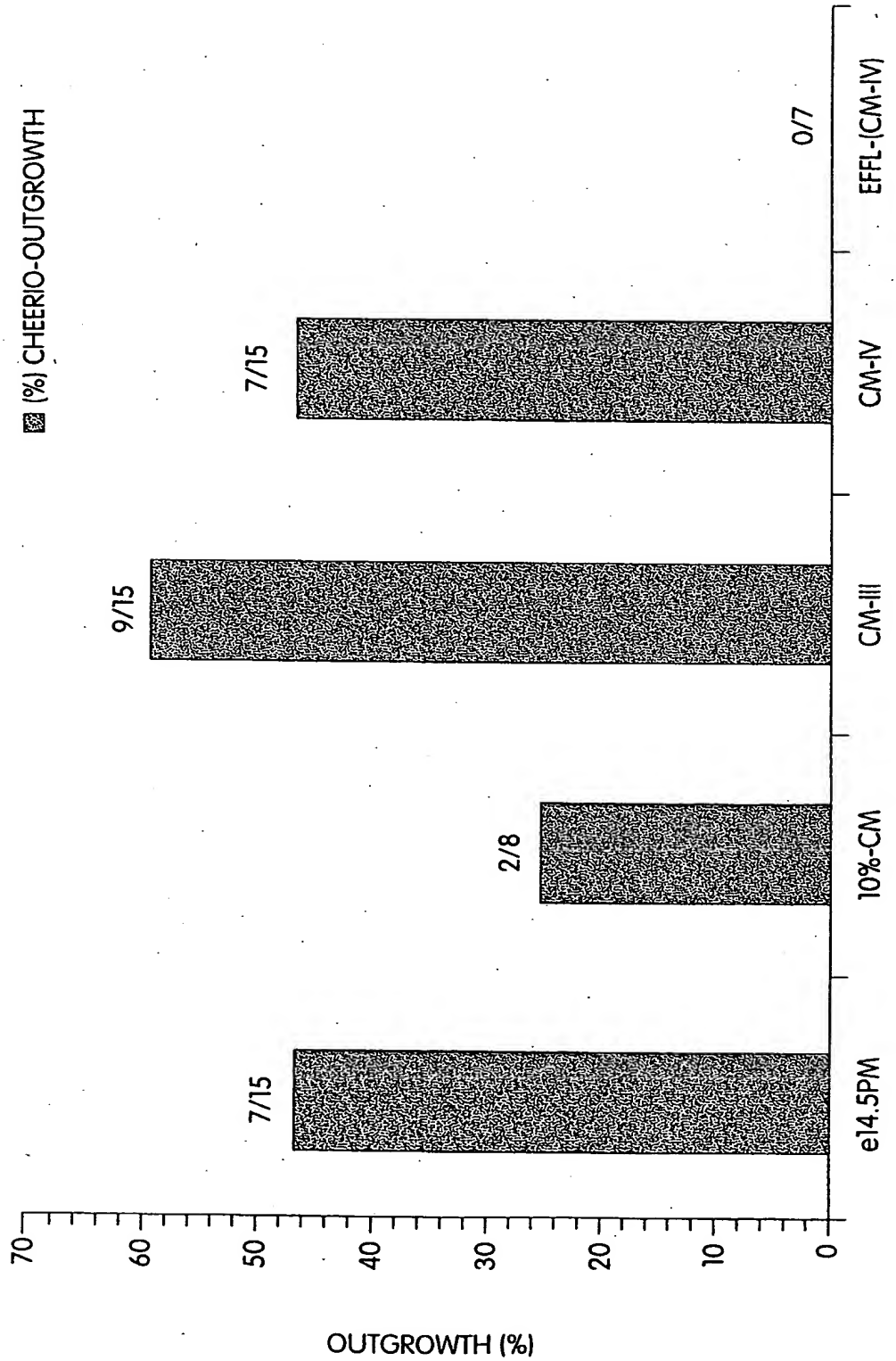
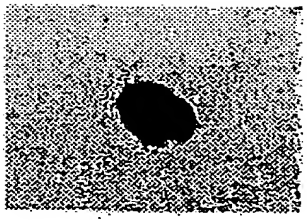
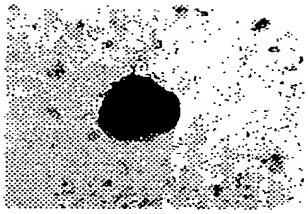

(%) CHEERIO-OUTGROWTH
7/15
9/15
2/8
7/15
0/7
e14.5PM
10%-CM
CM-III
CM-IV
EFFL-(CM-IV)
GROWTH CONDITIONS
OUTGROWTH (%)
0
10
20
30
40
50
60
70

Fig. 7



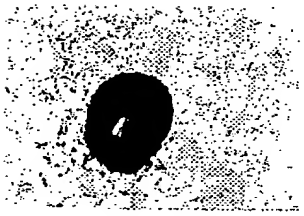
e14.5 PM
Positive Control

Fig. 8A



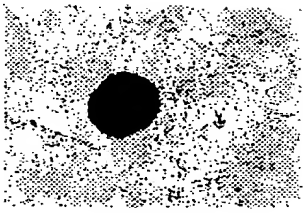
Clone 4

Fig. 8B



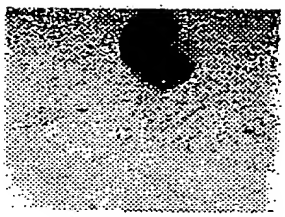
Clone 5

Fig. 8C



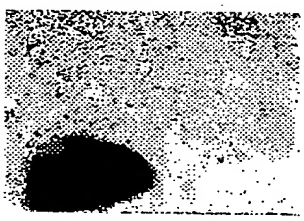
Clone 6

Fig. 8D



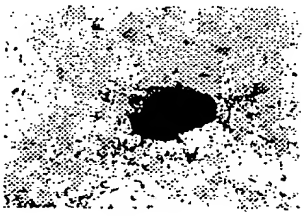
Clone 14

Fig. 8E



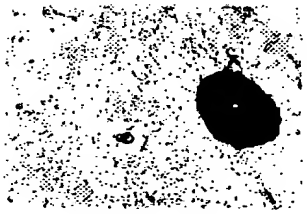
Clone 15

Fig. 8F



Clone 21

Fig. 8G



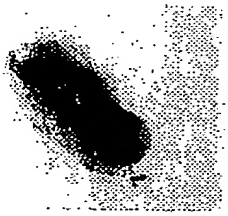
Clone 26

Fig. 8H

Negative Cell Lines

Clone 3

Fig. 8I



Clone 16

Fig. 8J

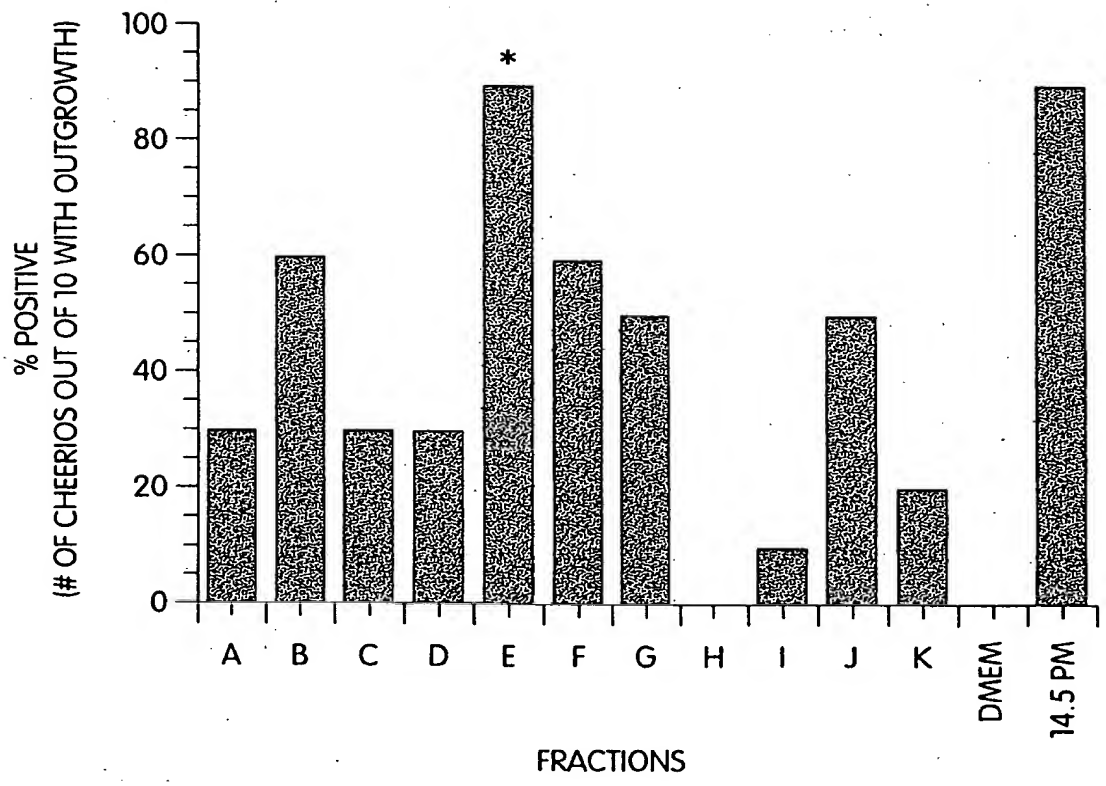
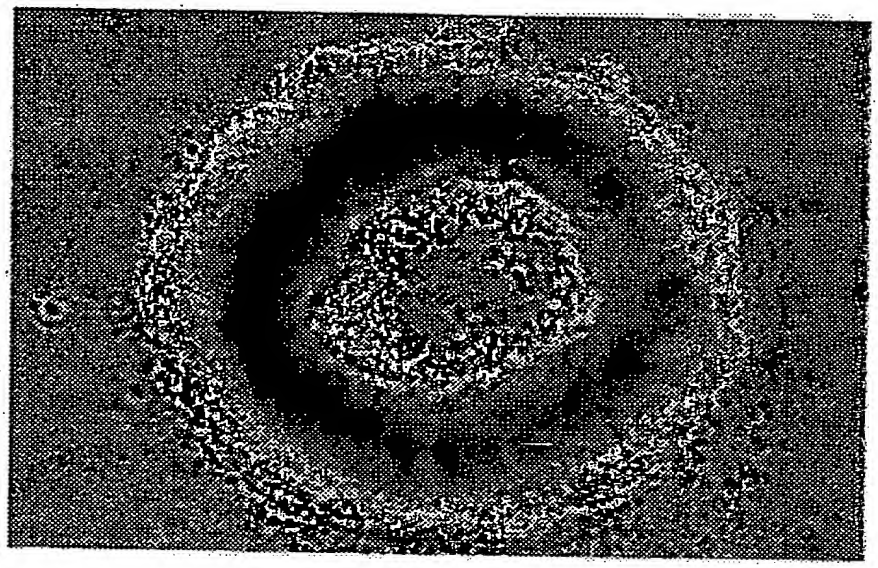
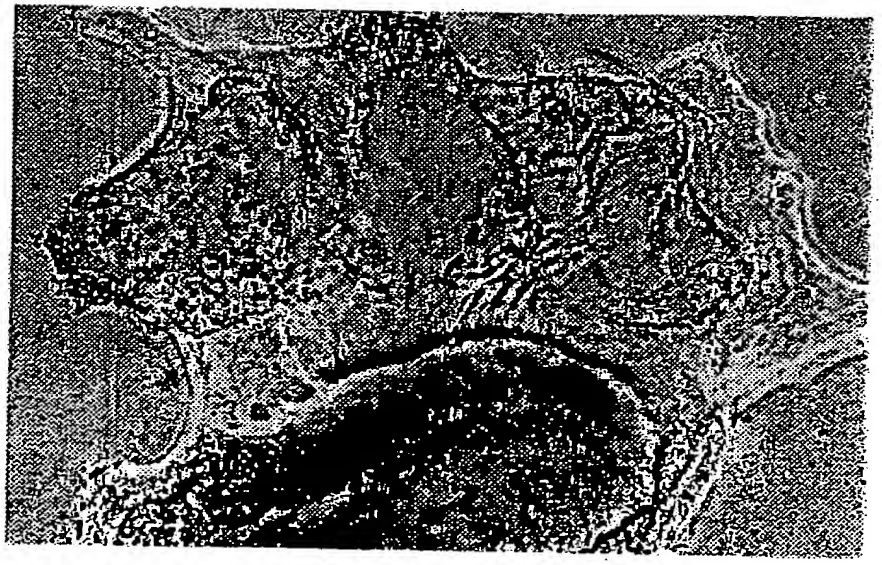

% POSITIVE
(# OF CHEERIOS OUT OF 10 WITH OUTGROWTH)
100
80
60
40
20
0
*
A
B
C
D
E
F
G
H
I
J
K
DMEM
14.5 PM
FRACTIONS

Fig. 9



DMEM



Fraction e

Fig. 10

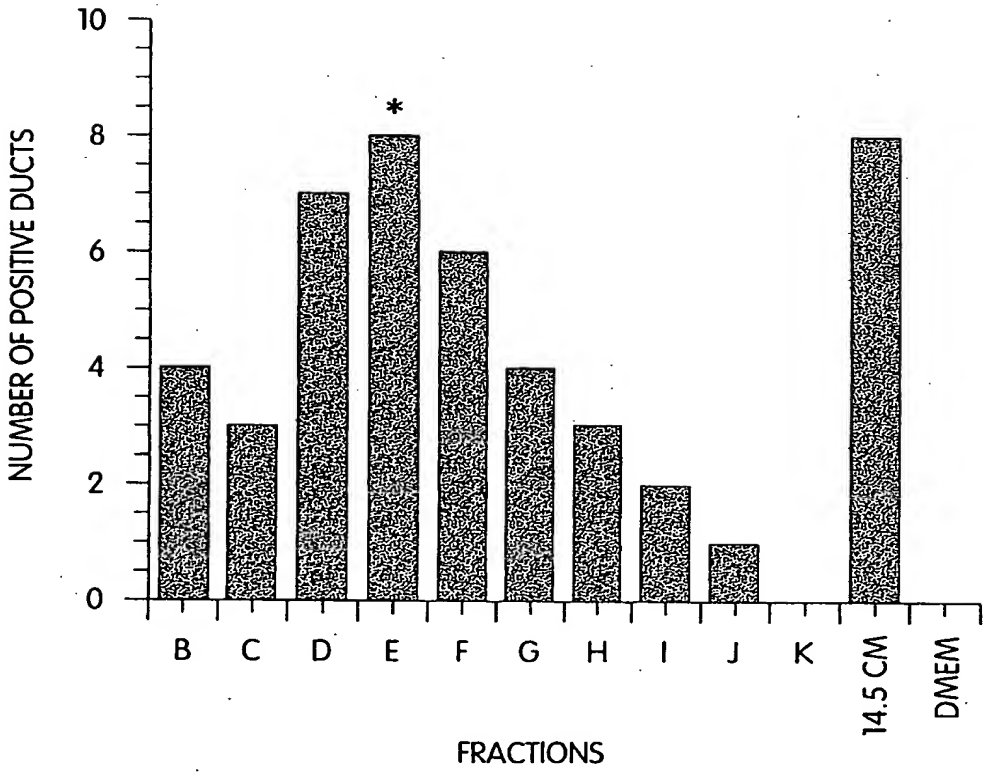

10
8
6
4
2
0
NUMBER OF POSITIVE DUCTS
*
B
C
D
E
F
G
H
I
J
K
14.5 CM
DMEM
FRACTIONS

Fig. 11

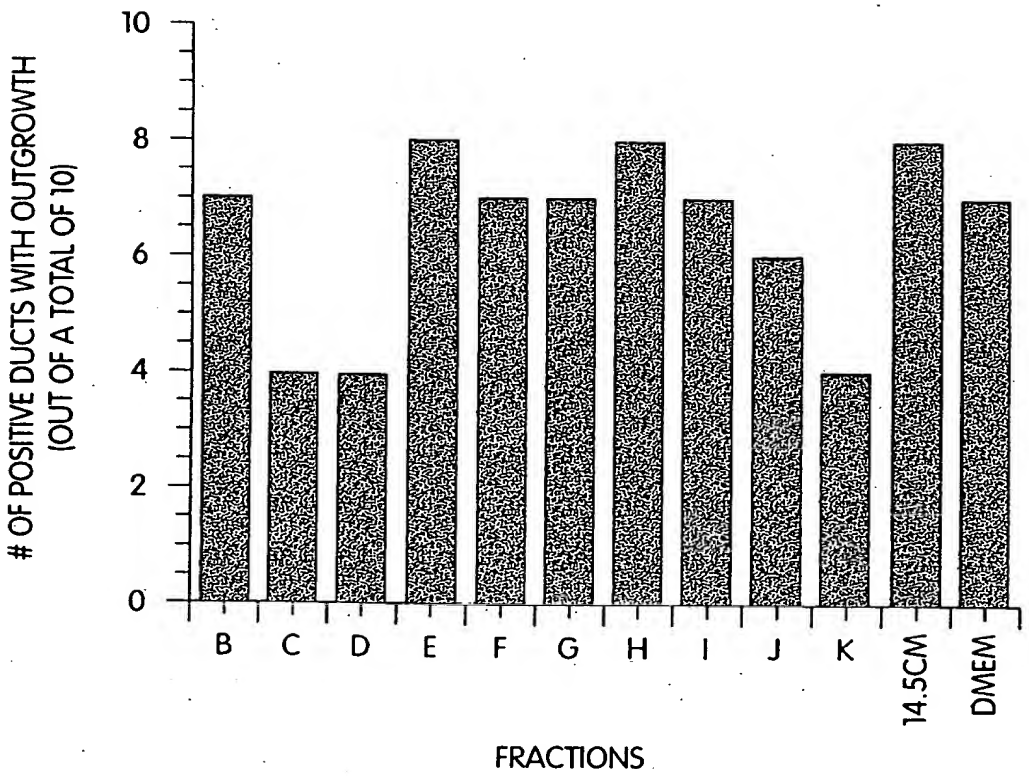

OF POSITIVE DUCTS WITH OUTGROWTH (OUT OF A TOTAL OF 10)
10
8
6
4
2
0
B
C
D
E
F
G
H
I
J
K
14.5CM
DMEM
FRACTIONS

Fig. 12

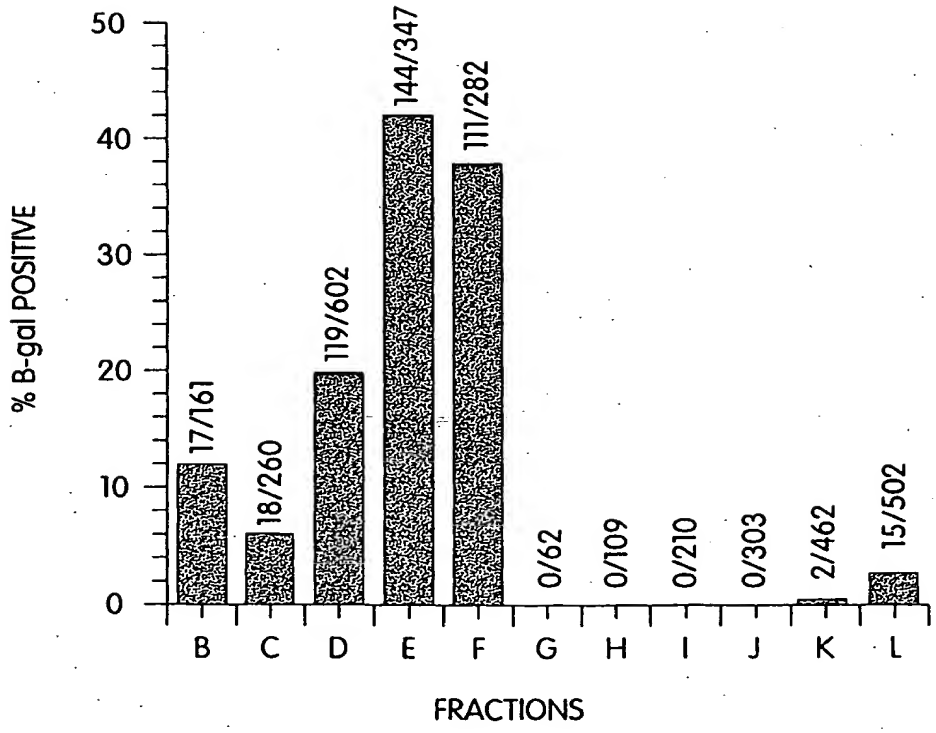
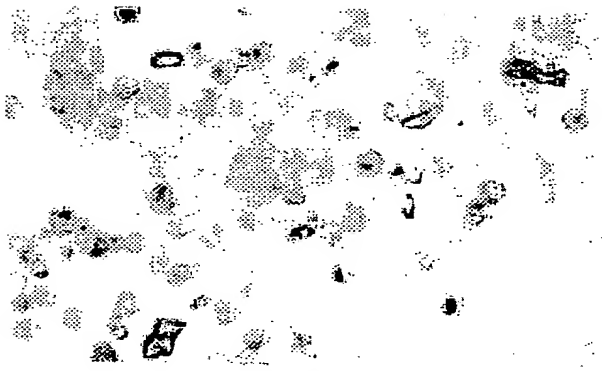
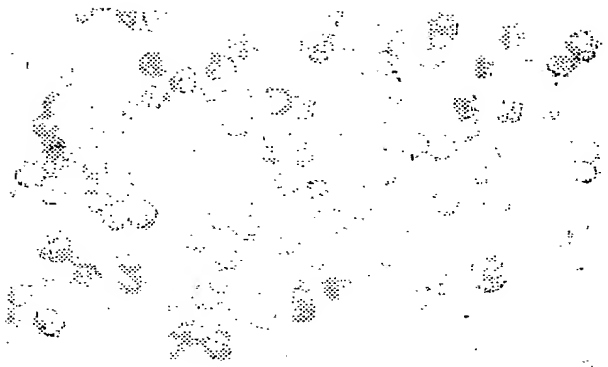

% B-gal POSITIVE
50
40
30
20
10
0
17/161
18/260
119/602
144/347
111/282
0/62
0/109
0/210
0/303
2/462
15/502
B
C
D
E
F
G
H
I
J
K
L
FRACTIONS

Fig. 13



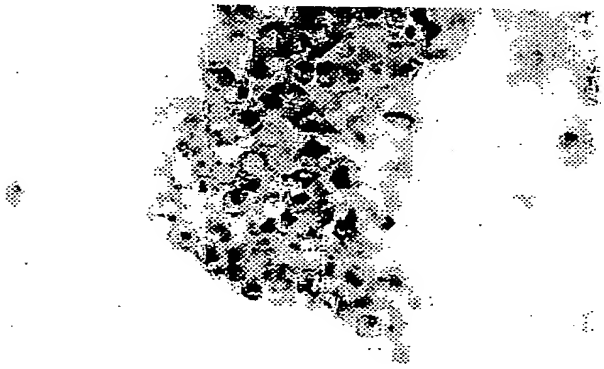
DMEM

Fig. 14A



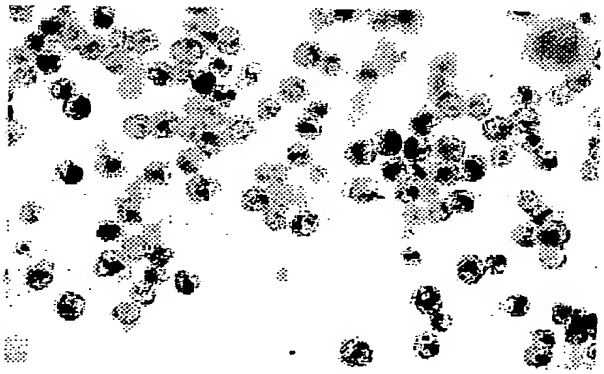
FRACTION i

Fig. 14C



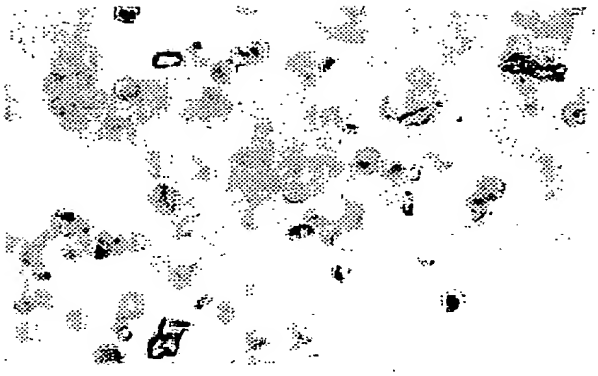
FRACTION d

Fig. 14B



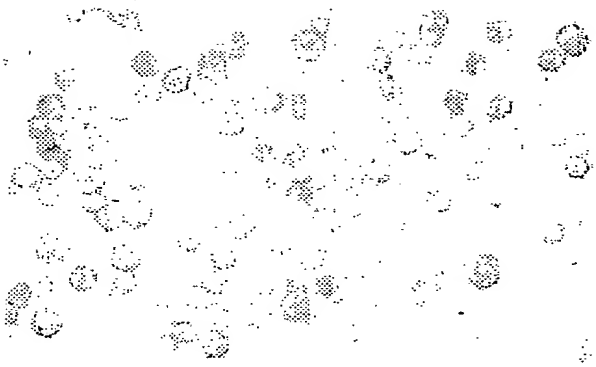
FRACTION e

Fig. 14D



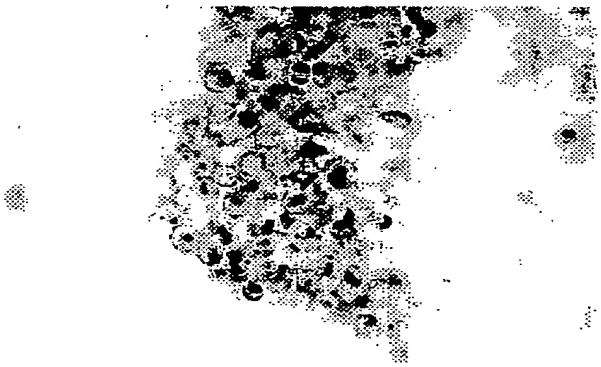
DMEM

Fig. 15A



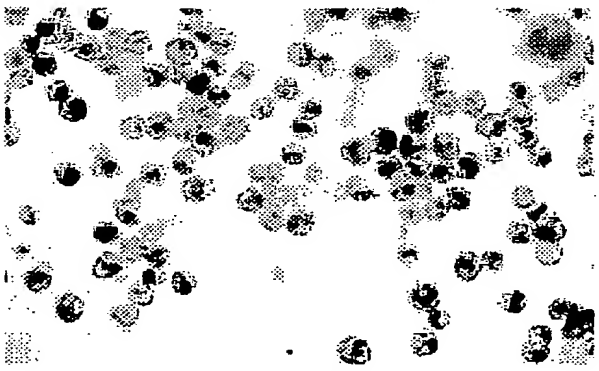
FRACTION i

Fig. 15C



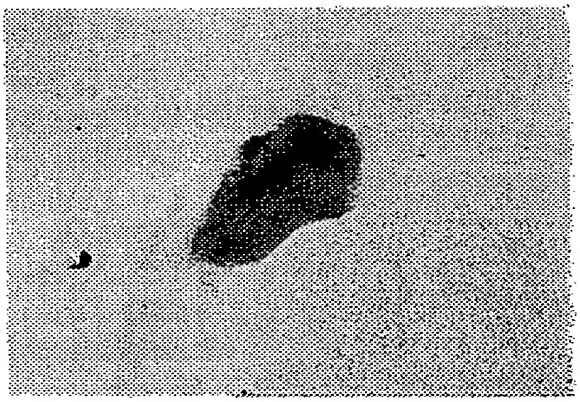
FRACTION d

Fig. 15B

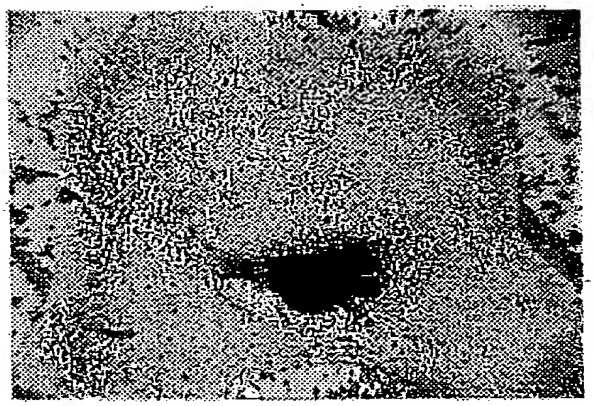


FRACTION e

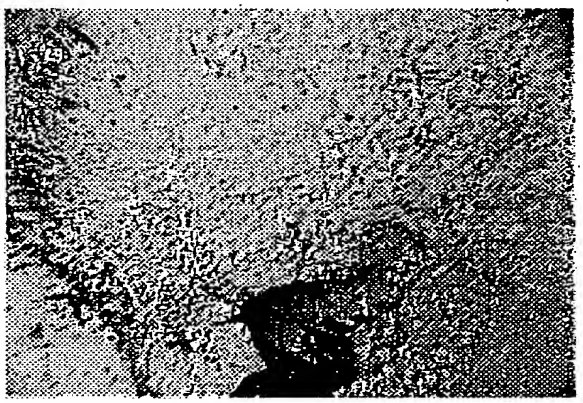
Fig. 15D



DMEM



14.5 PM CM



BOILED 14.5PM CM

Fig. 16

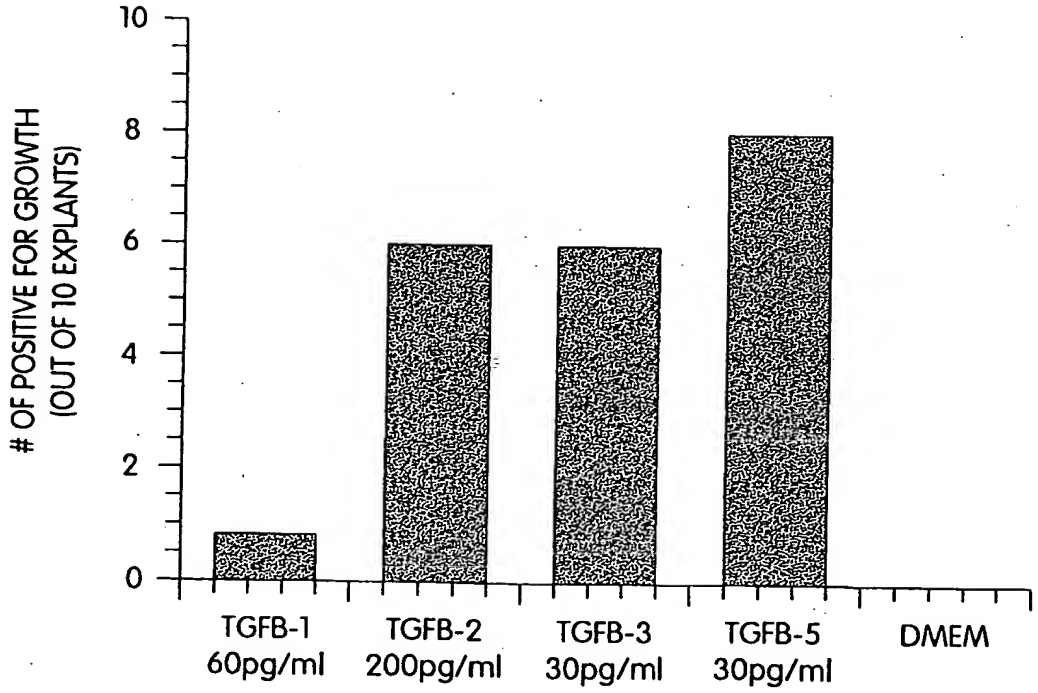

OF POSITIVE FOR GROWTH (OUT OF 10 EXPLANTS)
10
8
6
4
2
0
TGFB-1 60pg/ml
TGFB-2 200pg/ml
TGFB-3 30pg/ml
TGFB-5 30pg/ml
DMEM

Fig. 17

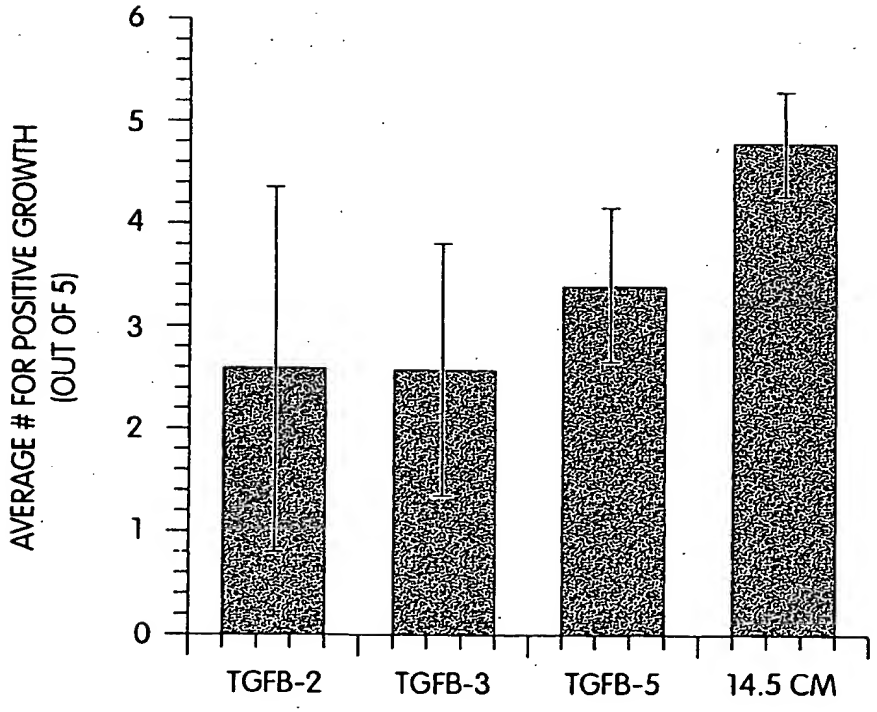

AVERAGE # FOR POSITIVE GROWTH
(OUT OF 5)
0
1
2
3
4
5
6
TGFB-2
TGFB-3
TGFB-5
14.5 CM

Fig. 18

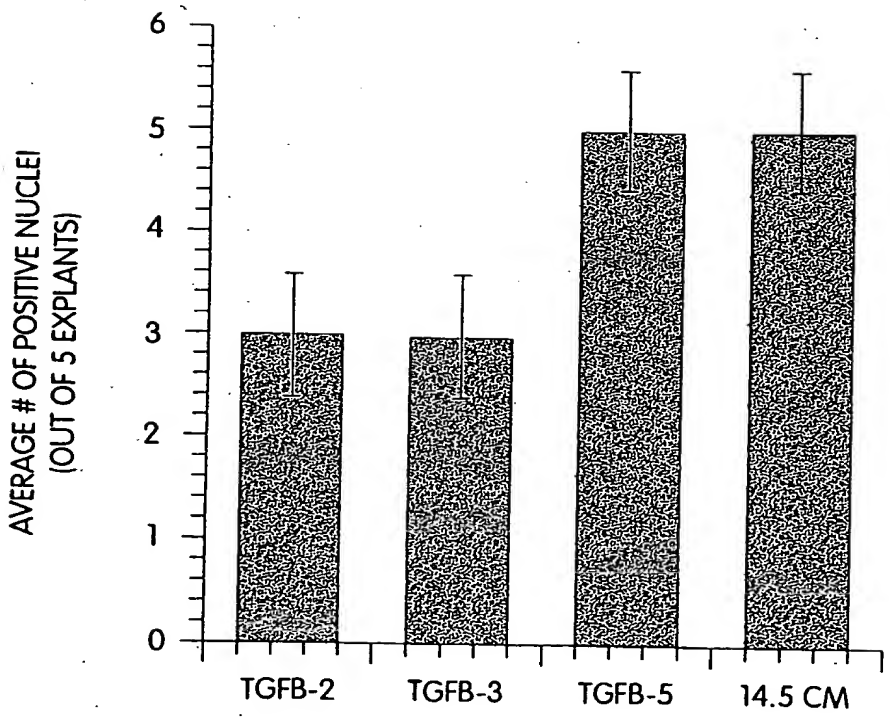

AVERAGE # OF POSITIVE NUCLEI
(OUT OF 5 EXPLANTS)
0
1
2
3
4
5
6
TGFB-2
TGFB-3
TGFB-5
14.5 CM

Fig. 19

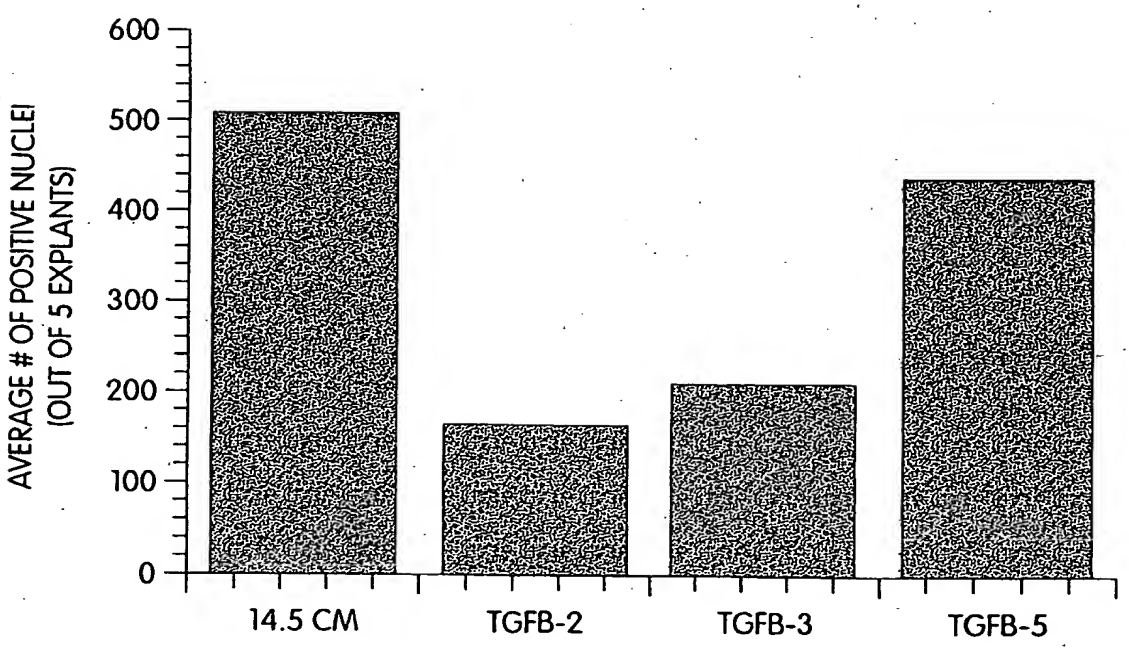

AVERAGE # OF POSITIVE NUCLEI (OUT OF 5 EXPLANTS)
600
500
400
300
200
100
0
14.5 CM
TGFB-2
TGFB-3
TGFB-5

Fig. 20

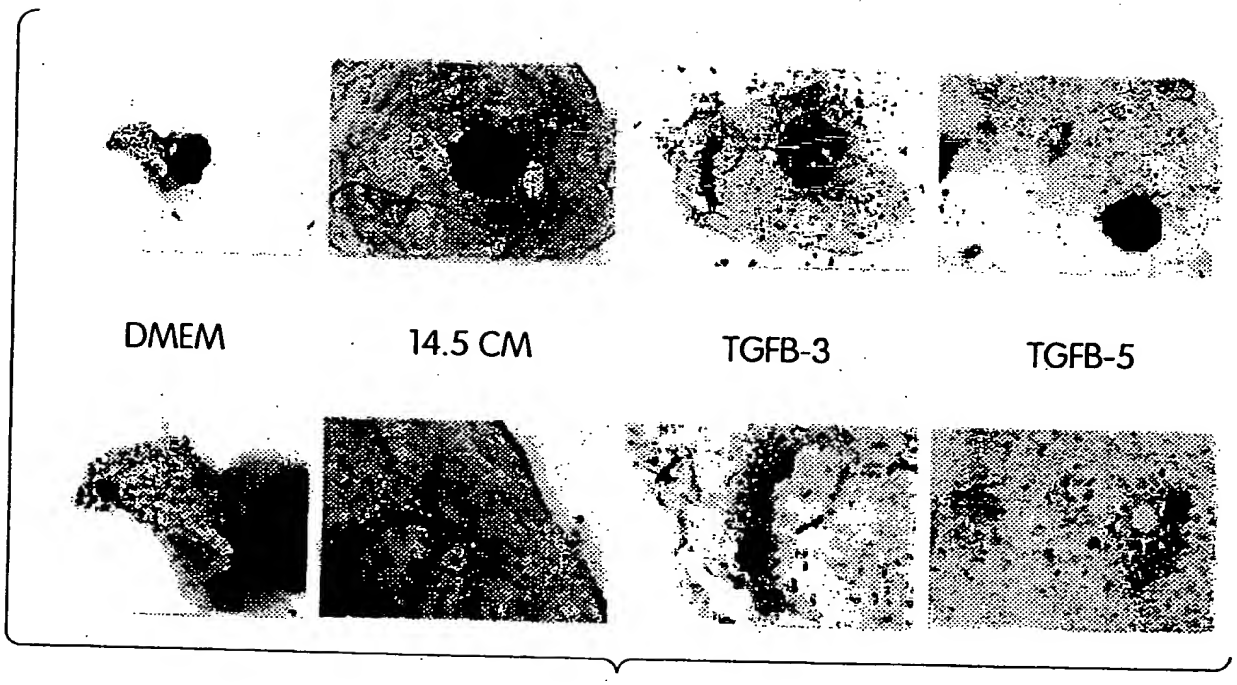


Fig. 21

Fig. 22A

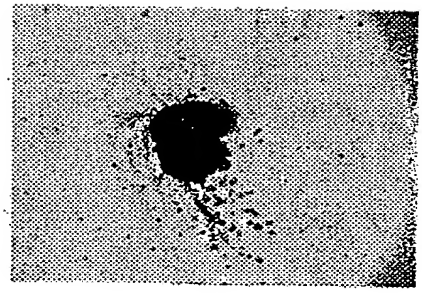


Fig. 22B

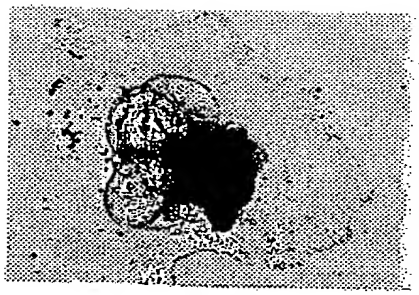


Fig. 22C

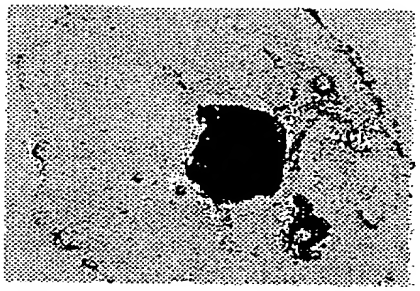


Fig. 22D

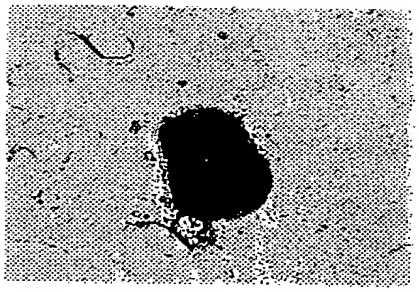


Fig. 22E

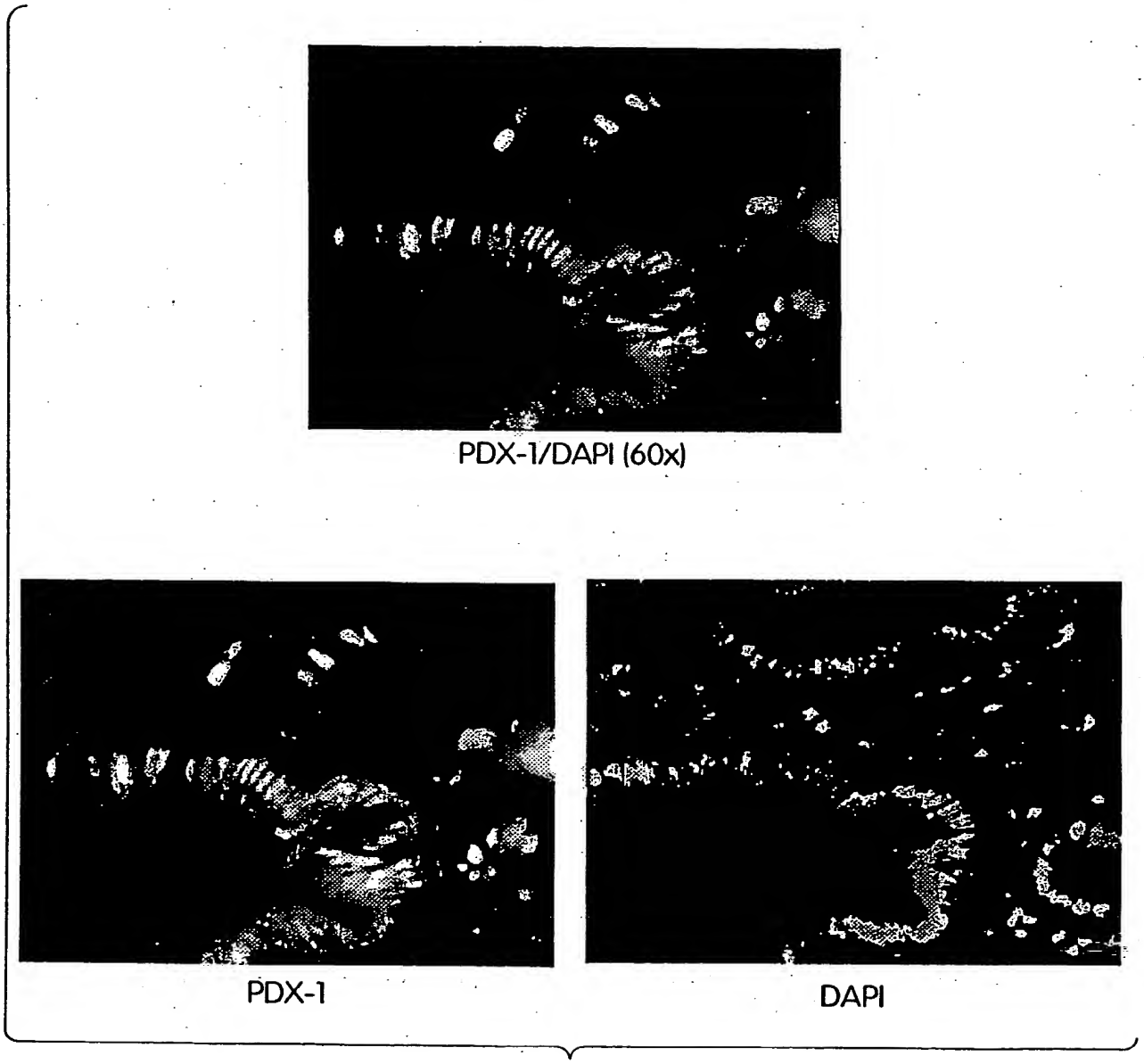


Fig. 23

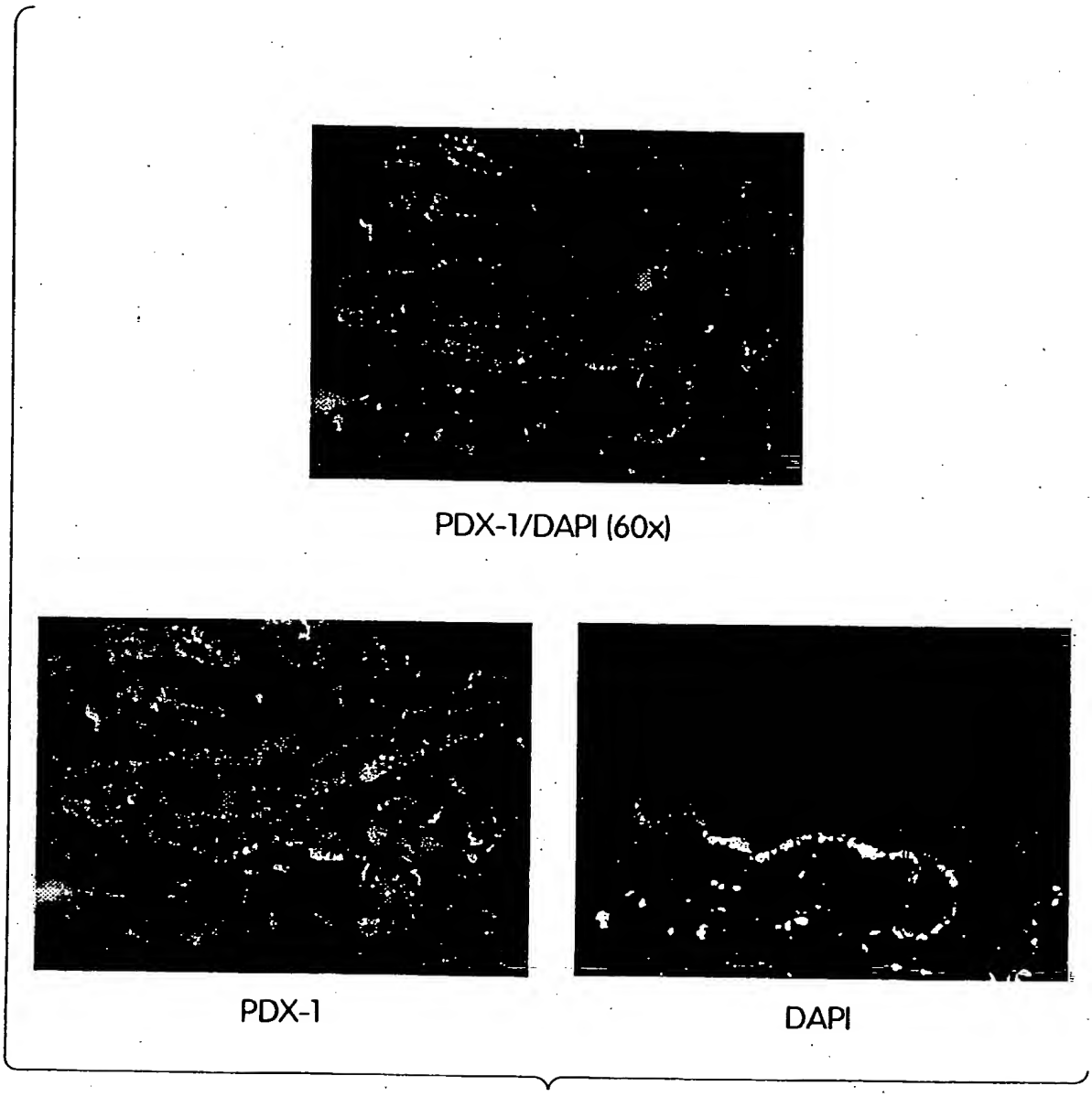


Fig. 24

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Guicherit, Oiven
Pang, Kevin
Wang, Monica

(ii) TITLE OF INVENTION: Pancreatic Ductal Trophic Factors, Uses and Composition Related Thereto

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Foley, Hoag & Eliot
(B) STREET: One Post Office Square
(C) CITY: Boston
(D) STATE: MA
(E) COUNTRY: USA
(F) ZIP: 02109

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: ASCII (text)

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 10-FEB-2000
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Vincent, Matthew P.
(B) REGISTRATION NUMBER: 36,709
(C) REFERENCE/DOCKET NUMBER: ONV-061.25

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (617) 832-1000
(B) TELEFAX: (617) 832-7000

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2754 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 504..1649

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 504..566

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 567..1649

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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AGCAAGCAGG GTTACAAGGG CCGTCAAATT ACATTTAGAG GGGGGAGTTG ACTGCATACA     120

AGGCACATAT TCTATAAGCT CTAGAAGTCA ACCCGGATCT CTCACACTGC TCAATGCAAC     180

TGCTTCATGA AGACAGGACA GCAACTTTCA AAACTGCTTT CAGCTTTTCT CACCAAGAGA     240

CCGTTCCATC CCTTTAACAT AAACACACAG TGTTCCAGTT GTAATTATGT TTTCGTTTAA     300

TGGTGGCAGC AAAACCTCTC CTCGTTTCTA AGTTGGCACA TGGTACTCTG AGCACAGCAC     360

ATATACAATG AATTGGGGCT GCTTAAACTG ACTGGAAGTG AATCTGAGCT GGTGCTGAGA     420

CTGTTGAAGG ACATAAAAGT AAAAGGAAAC TATTCTAAAA ACCTAAAGCC AGAAACTTGA     480

ATTTTGTTGT GGAACACTGC ATC ATG GAG GTT CTG TGG ATG CTG CTG GTG           530
                         Met Glu Val Leu Trp Met Leu Leu Val
                         -21 -20                 -15

CTA TTG GTC CTG CAT CTT TCC AGT TTA GCT ATG TCC TTG TCT ACC TGT        578
Leu Leu Val Leu His Leu Ser Ser Leu Ala Met Ser Leu Ser Thr Cys
      -10                 -5                  1

AAG GCT GTG GAT ATG GAA GAA GTC AGG AAG AGG AGA ATT GAA GCC ATC        626
Lys Ala Val Asp Met Glu Glu Val Arg Lys Arg Arg Ile Glu Ala Ile
  5                  10                  15                  20

AGG GGC CAA ATT CTC AGC AAG CTA AAA CTG GAC AAA ACA CCA GAT GTA        674
Arg Gly Gln Ile Leu Ser Lys Leu Lys Leu Asp Lys Thr Pro Asp Val
                 25                  30                  35

GAC AGC GAG AAG ATG ACA GTG CCC AGT GAA GCC ATC TTC CTT TAC AAT        722
Asp Ser Glu Lys Met Thr Val Pro Ser Glu Ala Ile Phe Leu Tyr Asn
             40                  45                  50

AGC ACC TTG GAG GTG ATC AGA GAA AAA GCA ACC AGA GAA GAA GAA CAT        770
Ser Thr Leu Glu Val Ile Arg Glu Lys Ala Thr Arg Glu Glu Glu His
         55                  60                  65

GTA GGG CAT GAT CAG AAC ATA CAG GAC TAT TAT GCA AAA CAA GTT TAC        818
Val Gly His Asp Gln Asn Ile Gln Asp Tyr Tyr Ala Lys Gln Val Tyr
     70                  75                  80

CGA TTT GAA AGC ATA ACC GAA CTG GAA GAC CAT GAA TTT AAG TTT AAA        866
Arg Phe Glu Ser Ile Thr Glu Leu Glu Asp His Glu Phe Lys Phe Lys
 85                  90                  95                  100

TTT AAT GCT TCG CAT GTG AGG GAG AAT GTA GGT ATG AAC TCT CTT CTG        914
Phe Asn Ala Ser His Val Arg Glu Asn Val Gly Met Asn Ser Leu Leu
                105                 110                 115

CAC CAT GCT GAG CTG CGC ATG TAC AAG AAG CAG ACA GAT AAA AAC ATG        962
His His Ala Glu Leu Arg Met Tyr Lys Lys Gln Thr Asp Lys Asn Met
            120                 125                 130

GAT CAA AGG ATG GAA CTA TTC TGG AAA TAC CAG GAA AAT GGG ACC ACT       1010
Asp Gln Arg Met Glu Leu Phe Trp Lys Tyr Gln Glu Asn Gly Thr Thr
        135                 140                 145

CAC TCA AGA TAC CTG GAG AGC AAG TAC ATT ACT CCA GTA ACA GAT GAT       1058
His Ser Arg Tyr Leu Glu Ser Lys Tyr Ile Thr Pro Val Thr Asp Asp
    150                 155                 160
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GAG TGG ATG TCA TTT GAT GTT ACA AAG ACT GTG AAT GAA TGG CTG AAA      1106
Glu Trp Met Ser Phe Asp Val Thr Lys Thr Val Asn Glu Trp Leu Lys
165                 170                 175                 180

CGT GCA GAG GAA AAT GAA CAA TTC GGG TTG CAG CCT GCC TGC AAA TGT      1154
Arg Ala Glu Glu Asn Glu Gln Phe Gly Leu Gln Pro Ala Cys Lys Cys
                185                 190                 195

CCC ACC CCA CAG GCC AAA GAT ATA GAC ATA GAA GGA TTT CCT GCT TTA      1202
Pro Thr Pro Gln Ala Lys Asp Ile Asp Ile Glu Gly Phe Pro Ala Leu
            200                 205                 210

CGT GGA GAC TTG GCG AGT TTG AGT TCC AAA GAA AAC ACG AAA CCC TAC      1250
Arg Gly Asp Leu Ala Ser Leu Ser Ser Lys Glu Asn Thr Lys Pro Tyr
        215                 220                 225

CTG ATG ATC ACT TCG ATG CCA GCT GAG CGT ATT GAC ACA GTT ACA AGC      1298
Leu Met Ile Thr Ser Met Pro Ala Glu Arg Ile Asp Thr Val Thr Ser
    230                 235                 240

TCA CGG AAA AAG CGA GGG GTT GGA CAG GAG TAT TGC TTT GGG AAC AAT      1346
Ser Arg Lys Lys Arg Gly Val Gly Gln Glu Tyr Cys Phe Gly Asn Asn
245                 250                 255                 260

GGG CCA AAC TGC TGT GTG AAA CCT CTT TAC ATA AAT TTT CGG AAG GAT      1394
Gly Pro Asn Cys Cys Val Lys Pro Leu Tyr Ile Asn Phe Arg Lys Asp
                265                 270                 275

CTA GGC TGG AAG TGG ATC CAT GAG CCT AAG GGA TAT GAA GCA AAT TAT      1442
Leu Gly Trp Lys Trp Ile His Glu Pro Lys Gly Tyr Glu Ala Asn Tyr
            280                 285                 290

TGT TTA GGA AAT TGT CCT TAC ATC TGG AGC ATG GAT ACT CAG TAC AGC      1490
Cys Leu Gly Asn Cys Pro Tyr Ile Trp Ser Met Asp Thr Gln Tyr Ser
        295                 300                 305

AAG GTG CTA TCA CTT TAT AAT CAG AAC AAT CCC GGT GCA TCT ATA TCT      1538
Lys Val Leu Ser Leu Tyr Asn Gln Asn Asn Pro Gly Ala Ser Ile Ser
    310                 315                 320

CCC TGC TGT GTT CCT GAT GTC TTG GAG CCA CTG CCA ATC ATT TAT TAT      1586
Pro Cys Cys Val Pro Asp Val Leu Glu Pro Leu Pro Ile Ile Tyr Tyr
325                 330                 335                 340

GTT GGC CGC ACT GCT AAA GTA GAG CAG CTC TCT AAT ATG GTG GTA AGG      1634
Val Gly Arg Thr Ala Lys Val Glu Gln Leu Ser Asn Met Val Val Arg
                345                 350                 355

TCT TGC AAC TGC AGC TGAGAAGAGC TTGGGGGCAG AAAGCAAAGC AAAGACATGG      1689
Ser Cys Asn Cys Ser
            360

TACAGAACCC ACAAACCACC TTACTGCTTT AACCAGTGTC GAAGAAGGAG ACCATGTTGT    1749

CAAGTCATGA GGATCGATGA ACCCGTTAAT TCTACATCTG AAAAGAGCAA ATGCTGAAAG    1809

CAGTTACTAA CTTCACAGCC CACACATGGA TTTAGGATTC TTCTACAGCA ATACACCTTT    1869

GGTTACAATA TCTGTATTCC TGCACTTCGC ACCTCAGTTA CTGTATCAAG TTTTTAGCTC    1929

TCTGCTACAG CTTTTATGAA AGTGCTTCCT TGCCAAAGGT TATGGACAAA TCTTAAGTTT    1989

TGGCAACAGC AAAATCTGGG TTCAAGGTAA AGTCACTCAT CTGAAAGCAT CCAGTCCATC    2049

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CATTCAAAAA ATGCTGACAA GAATGCCTTA CAGAGAAGTT AACCCACTCA ATGCTGAGCA 2289

CAGATTGGAG AATCACAAGG CTCTTTATAG ATTGTGTATT TATTAACATA TATAATTGCC 2349

ACATAAAAGC TCAAGCATTT CGTATTAGAC ATATTAATGA TAAAAAAATA TTCACAACTG 2409

TTTTTGATAA AACATGGGGG TTCCCATGTT CATTTCTACT ATCCCAACTT GTAGAGCTGT 2469

GAATGTGTTT AAAAGGAAAC AAAGACAATG ATTTACAAAT CCAGAATATA GGATCATCAG 2529

AAAAGAAACA TATGACTCTG CTGCAGGACT GACCTGTCAG ATATAACCGG ATGAAATACA 2589

GAAAGCCATG TGCAGGTACC CACTGAACAA AGAAGGGGCT AATAATTCCT GTTTAAATGA 2649

GAAGGGTTTT TTTTTTACCT TTTTAAATGG TCTTATTTTA TATAGTTTTT TTTTTAATAT 2709

TTAAAAGGTT TTGTTTTAAT GAGGAAAAAT AAAAAAGGTA CATAC 2754

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 382 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Glu Val Leu Trp Met Leu Leu Val Leu Leu Val Leu His Leu Ser
-21 -20 -15 -10

Ser Leu Ala Met Ser Leu Ser Thr Cys Lys Ala Val Asp Met Glu Glu
-5 1 5 10

Val Arg Lys Arg Arg Ile Glu Ala Ile Arg Gly Gln Ile Leu Ser Lys
15 20 25

Leu Lys Leu Asp Lys Thr Pro Asp Val Asp Ser Glu Lys Met Thr Val
30 35 40

Pro Ser Glu Ala Ile Phe Leu Tyr Asn Ser Thr Leu Glu Val Ile Arg
45 50 55

Glu Lys Ala Thr Arg Glu Glu Glu His Val Gly His Asp Gln Asn Ile
60 65 70 75

Gln Asp Tyr Tyr Ala Lys Gln Val Tyr Arg Phe Glu Ser Ile Thr Glu
80 85 90

Leu Glu Asp His Glu Phe Lys Phe Lys Phe Asn Ala Ser His Val Arg
95 100 105

Glu Asn Val Gly Met Asn Ser Leu Leu His His Ala Glu Leu Arg Met
110 115 120

Tyr Lys Lys Gln Thr Asp Lys Asn Met Asp Gln Arg Met Glu Leu Phe
125 130 135

Trp Lys Tyr Gln Glu Asn Gly Thr Thr His Ser Arg Tyr Leu Glu Ser

140 145 150 155

Lys Tyr Ile Thr Pro Val Thr Asp Asp Glu Trp Met Ser Phe Asp Val
160 165 170

Thr Lys Thr Val Asn Glu Trp Leu Lys Arg Ala Glu Glu Asn Glu Gln
175 180 185

Phe Gly Leu Gln Pro Ala Cys Lys Cys Pro Thr Pro Gln Ala Lys Asp
190 195 200

Ile Asp Ile Glu Gly Phe Pro Ala Leu Arg Gly Asp Leu Ala Ser Leu
205 210 215

Ser Ser Lys Glu Asn Thr Lys Pro Tyr Leu Met Ile Thr Ser Met Pro
220 225 230 235

Ala Glu Arg Ile Asp Thr Val Thr Ser Ser Arg Lys Lys Arg Gly Val
240 245 250

Gly Gln Glu Tyr Cys Phe Gly Asn Asn Gly Pro Asn Cys Cys Val Lys
255 260 265

Pro Leu Tyr Ile Asn Phe Arg Lys Asp Leu Gly Trp Lys Trp Ile His
270 275 280

Glu Pro Lys Gly Tyr Glu Ala Asn Tyr Cys Leu Gly Asn Cys Pro Tyr
285 290 295

Ile Trp Ser Met Asp Thr Gln Tyr Ser Lys Val Leu Ser Leu Tyr Asn
300 305 310 315

Gln Asn Asn Pro Gly Ala Ser Ile Ser Pro Cys Cys Val Pro Asp Val
320 325 330

Leu Glu Pro Leu Pro Ile Ile Tyr Tyr Val Gly Arg Thr Ala Lys Val
335 340 345

Glu Gln Leu Ser Asn Met Val Val Arg Ser Cys Asn Cys Ser
350 355 360